For efficient cell entry, SARS-CoV-2 spike protein needs to be cleaved by cellular proteases. Here, we present a comprehensive protocol to assess SARS-CoV-2 spike protein cleavage in viral supernatants from SARS-CoV-2-infected cells. We also include a previous step of SARS-CoV-2 isolation from nasopharyngeal swabs of patients with COVID-19. We optimized the procedures to enhance successful viral isolation and specific spike detection. This protocol facilitates the evaluation of the role of spike mutations in spike protein processing.

Publisher’s note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.
Protocol to isolate and assess spike protein cleavage in SARS-CoV-2 variants obtained from clinical COVID-19 samples

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SUMMARY
For efficient cell entry, SARS-CoV-2 spike protein needs to be cleaved by cellular proteases. Here, we present a comprehensive protocol to assess SARS-CoV-2 spike protein cleavage in viral supernatants from SARS-CoV-2-infected cells. We also include a previous step of SARS-CoV-2 isolation from nasopharyngeal swabs of patients with COVID-19. We optimized the procedures to enhance successful viral isolation and specific spike detection. This protocol facilitates the evaluation of the role of spike mutations in spike protein processing. For complete details on the use and execution of this protocol, please refer to Escalera et al. (2022).

BEFORE YOU BEGIN
Institutional permissions
SARS-CoV-2 is a biosafety level 3 (BSL-3) pathogen and therefore, an appropriate facility and training for researchers is needed. Ensure the institution has the appropriate licenses and biosafety approvals before starting the experiments.

Alternatives: This protocol describes the specific steps to assess spike protein cleavage in supernatants from Vero-TMPRSS2 cells infected with SARS-CoV-2 variants isolated from nasopharyngeal swabs of COVID-19 infected patients. We have specifically used Vero-TMPRSS2 cells in these experiments to study the role and usage of TMPRSS2 protease in spike protein cleavage by the different SARS-CoV-2 variants. However, we have also used this protocol to study spike protein processing in VeroE6, Caco-2 and human pneumocyte-like cells using both viral supernatants and cell extracts.

Confirmation of SARS-CoV-2 infection by RT-qPCR
© Timing: 4 h
CRITICAL: Nasopharyngeal swabs must be collected by trained professionals. Upon collection, swabs must be placed in viral transport media and store at 4°C (not longer than 4 days) or at −80°C to ensure viral viability.

Note: As an alternative, saliva samples from acute COVID-19 patients could be collected following same procedure. We have used commercial flocked swabs containing universal viral transport media (see key resources table). However, other collection methods and viral transport solutions can be used. If prepare in-house, please follow the CDC standard operating procedure (SOP) to make viral transport media (CDC Protocol for Viral Transport Media).

1. Extract the RNA from SARS-CoV-2 viruses present in the viral transport media using an RNA extraction kit following manufacturer’s indications.

Note: Nasopharyngeal swabs usually contain ~2 mL of viral transport media. For RNA extraction, we use the E.Z.N.A Viral RNA kit which requires 150 μL of viral sample. To ensure optimal viral RNA isolation, we recommend eluting the viral RNA in 30 μL of RNase free water provided in the kit.

2. Perform RT-qPCR to confirm positivity of sample and to determine the viral load of the SARS-CoV-2 variant following manufacturer’s instructions.

Note: We use the CDC 2019-nCoV real-time RT-qPCR protocol with two 2019-nCoV-specific primer sets (N1, N2) and one primer set to detect host cellular RNaseP as control for cellular input (see key resources table (Escalaera et al., 2022)). Prepare three PCR reaction master mix using each of the primer sets (see Table below). Each sample should be amplified in duplicates for each primer set.

| Reagent                        | Amount |
|--------------------------------|--------|
| RNA template                   | 6.25 μL|
| Reverse transcriptase          | 0.25 μL|
| Primer/Probe N1/ N2/ RNaseP    | 2.5 μL |
| Mastermix                      | 5 μL   |
| ddH₂O                          | 11 μL  |
| **Total**                      | 25 μL  |

| Steps                          | Temperature | Time  | Cycles |
|--------------------------------|-------------|-------|--------|
| Reverse Transcription (RT)     | 50°C        | 20 min| 1      |
| RT heat inactivation           | 95°C        | 1 s   | 1      |
| DNA polymerase activation      | 95°C        | 5 min | 1      |
| Denaturalization               | 95°C        | 15 s  | 40     |
| Annealing/Extension            | 60°C        | 45 s  |        |
| Hold                           | 4°C         | Forever|        |

△ CRITICAL: A positive control is required to determine the limit of detection for SARS-CoV-2 by RT-qPCR. For successful viral isolation, samples should have low Ct values, ≤25 has shown the best results (Aydillo et al., 2020). Additionally, nasopharyngeal swab must be sequenced, preferably by next generation sequencing techniques such as Illumina or Minion technologies. This step will allow to have a reference genome to compare with the sequence from the viral stocks generated in the next steps. Sequence will be used
to identify potential mutations that may emerge from cell culture. For further reference see methods (Escalera et al., 2022; Aydillo et al., 2020; Gonzalez-Reiche et al., 2020).

Plate Vero-TMPRSS2 cells

**Timing:** 1 h

For this protocol, it will be needed to use Vero-TMPRSS2 cells in anticipation for viral isolation, plaque purification or plaque assay.

3. The day before performing these experiments, seed approximately $3.2 \times 10^5$ Vero-TMPRSS2 cells/well using 1 mL/well of growth media in a 12 well-plate or $8.5 \times 10^5$ Vero-TMPRSS2 cells/well using 2 mL/well of growth media in a 6 well-plate. Maintain cells at 37°C in 5% CO₂ overnight (16–18 h).

**Note:** After thawing Vero-TMPRSS2 cells, allow at least 3 passages before using them in the experiments. Since many steps in this protocol require plating Vero-TMPRSS2 cells the day before, maintain cells in culture up to passage number 20.

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| SARS-CoV-2 NP antibody (1:1000) | Center for Therapeutic Antibody Development (CTAD), Icahn School of Medicine at Mount Sinai | Cat# NP1C7C7 |
| SARS-CoV-2 Spike Protein S2 mAb (1A9) (1:3000) | Thermo Fisher Scientific | Thermo Fisher Scientific Cat# MAS-35946, RRID: AB_2866558 |
| SARS-CoV-2 Nucleocapsid Protein antibody (1:2000) | Novus Biologicals | Novus Cat# NB100-56576, RRID: AB_838838 |
| Anti-mouse IgG-HRP antibody (1:5000) | Abcam | Abcam Cat# ab6823, RRID: AB_955395 |
| Anti-rabbit IgG-HRP antibody (1:3000) | Kindle Biosciences | Kindle Biosciences Cat# R1006, RRID: AB_2800464 |
| Experimental models: Cell lines | | |
| VeroE6-TMPRSS2 (Epithelial kidney monkey cells expressing human TMPRSS2 protease) | BPS Bioscience | Cat# 78081 |
| Oligonucleotides | | |
| RT-qPCR primers RUO Kit | Integrated DNA Technologies | Cat# 10006713 |
| Software and algorithms | | |
| ImageJ | NIH | [https://imagej.nih.gov/ij/download.html](https://imagej.nih.gov/ij/download.html) |
| Graphpad Prism | GraphPad | [https://www.graphpad.com/scientific-software/prism/](https://www.graphpad.com/scientific-software/prism/) |
| Other | | |
| Flocked swabs | BD | Cat# 220531 |
| E.Z.N.A Viral RNA kit | Omega Bio-tek | Cat# R6874 |
| QuantiFast Pathogen RT-PCR + IC Kit | QIAGEN | Cat# 211454 |
| Dulbecco’s modified Eagle’s medium (DMEM) with glucose, L-glutamine, and sodium pyruvate | Corning | Cat# 10-017-CV |
| Fetal bovine serum (FBS) | Gibco | Cat# 16140071 |
| Non-essential amino acids | Corning | Cat# 25-025-CI |
| HEPES | Gibco | Cat# 15630080 |
| Penicillin and streptomycin (100 UI/mL) | Corning | Cat# 30-002-CI |

(Continued on next page)
## MATERIALS AND EQUIPMENT

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Normocin (50 mg/mL) | InvivoGen | Cat# ant-nr-1 |
| Puromycin (10 mg/mL) | InvivoGen | Cat# ant-pr-1 |
| Amphotericin B (250 µg/mL) | Gibco | Cat# 15290018 |
| Cryotube vials | Thermo Scientific | Cat# 363401 |
| 10X MEM | Lonza | Cat# 12684F |
| L-Glutamine (100 UI/mL) | Gibco | Cat# 25030081 |
| 35% Bovine Albumin | MP Bio | Cat# 08810061 |
| NaHCO₃ | Thermo Scientific | Cat# 144-55-8 |
| Oxoid agar | Thermo Scientific | Cat# LP0028B |
| EDTA-free protease inhibitor cocktail | Roche | Cat# 04693132001 |
| 10% SDS | Invitrogen | Cat# AM9822 |
| 10% TGX gels | Bio-Rad | Cat# 4561036 |
| Loading buffer 5X | Thermo Scientific | Cat# 39000 |
| Prestained Protein Ladder, 10–250 kDa | Thermo Scientific | Cat# 26619 |
| 10X Tris/Glycine/SDS Running buffer | Bio-Rad | Cat# 1610772 |
| Polyvinylidene fluoride (PVDF) membranes | Bio-Rad | Cat# 704156 |
| BIO-RAD semi-dry transfer system | Bio-Rad | Cat# 1704150 |
| 10X TBS buffer (pH 7.4) | Boston BioProducts | Cat# BM-301 |
| Non-fat dry milk | Boston BioProducts | Cat# P-1400 |
| Tween-20 | Thermo Scientific | Cat# BP337-100 |
| SuperSignal™ West Pico PLUS Chemiluminescent Substrate | Thermo Scientific | Cat# 34577 |
| Pierce™ ECL Western Blotting Substrate | Thermo Scientific | Cat# 32106 |

### Growth media

| Reagent | Final concentration | Amount |
|---------|---------------------|--------|
| DMEM with glucose, L-glutamine, and sodium pyruvate | N/A | 500 mL |
| Fetal bovine serum (FBS) | 10% (v/v) | 50 mL |
| Non-essential amino acids | 1% (v/v) | 5 mL |
| Penicillin and streptomycin (100 UI/mL) | 1% (v/v) | 5 mL |
| Normocin (50 mg/mL) | 100 µg/mL | 1 mL |
| Puromycin (10 mg/mL) | 3 µg/mL | 150 µL |
| Total | N/A | 561.150 mL |

Keep at 4°C. Store for up to one month.

### Infection media

| Reagent | Final concentration | Amount |
|---------|---------------------|--------|
| DMEM with glucose, L-glutamine, and sodium pyruvate | N/A | 500 mL |
| Fetal bovine serum (FBS) | 2% (v/v) | 10 mL |
| Non-essential amino acids | 1% (v/v) | 5 mL |
| HEPES | 1% (v/v) | 5 mL |
| Penicillin and streptomycin (100 UI/mL) | 1% (v/v) | 5 mL |
| Normocin (50 mg/mL) | 100 µg/mL | 1 mL |
| Total | N/A | 526 mL |

Keep at 4°C. Store for up to one month.
### Isolation media

| Reagent       | Final concentration | Amount  |
|---------------|---------------------|---------|
| Infection media | N/A                 | 50 mL   |
| Amphotericin B | 0.5 µg/mL           | 100 µL  |
| Total         | N/A                 | 50.1 mL |

Add amphotericin before use. Always prepare and use fresh.

### Minimum essential media (MEM)

| Reagent                              | Final concentration | Amount  |
|--------------------------------------|---------------------|---------|
| 10× MEM                              | 2×                  | 100 mL  |
| Penicillin and streptomycin (100 UI/mL) | 2% (v/v)             | 10 mL   |
| L-Glutamine (100 UI/mL)              | 2% (v/v)             | 10 mL   |
| 35% Bovine Albumin                   | 0.4% (v/v)           | 6 mL    |
| HEPES                                | 2% (v/v)             | 10 mL   |
| 5% NaHCO₃                            | 0.24% (v/v)          | 24 mL   |
| Total                                | N/A                 | 500 mL  |

Keep at 4°C. Store for up to one month.

### Overlay medium

| Reagent      | Final concentration | Amount  |
|--------------|---------------------|---------|
| MEM          | N/A                 | 31.5 mL |
| FBS          | 2%                  | 1 mL    |
| 2% Oxoid agar | 0.7%                | 17.5 mL |
| Total        | N/A                 | 50 mL   |

Always prepare and use fresh.

### Lysis buffer

| Reagent                              | Final concentration | Amount  |
|--------------------------------------|---------------------|---------|
| RIPA buffer                          | 1×                  | 10 mL   |
| EDTA-free protease inhibitor cocktail (tablets) | N/A                 | 1 tablet|
| Total                                | N/A                 | 10 mL   |

Reagents are kept at 4°C. Always prepare and use fresh.

To 9 mL of the buffer, add 1 mL of 10% SDS to a final concentration of 1%

**Note:** Prepare fresh before use, storage not recommended.

### Running buffer

| Reagent                              | Final concentration | Amount  |
|--------------------------------------|---------------------|---------|
| 10× Tris/Glycine/SDS                 | 1×                  | 100 mL  |
| ddH₂O                                | N/A                 | 900 mL  |
| Total                                | N/A                 | 1 L     |

Keep at room temperature (RT, 15°C–25°C). Always prepare and use fresh.

### 10× TBS buffer (pH 7.4)

| Reagent      | Final concentration | Amount  |
|--------------|---------------------|---------|
| Tris Base/Tris-HCl | 500 mM               | 78 g    |
| NaCl         | 1.5 M               | 87.7 g  |

(Continued on next page)
**Note:** We use a commercial 10× TBS buffer (pH 7.4) (see key resources table). However, in-house preparation can be made following the recipe above.

### Wash buffer (TBST)

| Reagent       | Final concentration | Amount |
|---------------|---------------------|--------|
| 10× TBS       | 1×                  | 100 mL |
| Tween-20      | 0.1% (v/v)          | 1 mL   |
| ddH₂O         | N/A                 | 900 mL |
| Total         | N/A                 | 1 L    |

Store at RT for several months.

### Other reagents

| Reagent                  | Final concentration                                                                 |
|--------------------------|--------------------------------------------------------------------------------------|
| 5% NaHCO₃                | 2.5 g NaHCO₃ in 50 mL ddH₂O                                                          |
| 2% Oxoid agar            | 10 g Oxoid agar in 500 mL ddH₂O                                                      |
| Blocking solution        | 1× TBST and 5% non-fat dry milk                                                     |
| Antibody dilution buffer | 1× TBST and 3% non-fat dry milk                                                     |

Keep at RT. Always prepare and use fresh.

### STEP-BY-STEP METHOD DETAILS

**Isolation of SARS-CoV-2 variants from nasopharyngeal swabs**

**Timing:** 4 days to 1 week

This step describes how to isolate and obtain SARS-CoV-2 virus from a nasopharyngeal swab previously collected from a COVID-19 infected individual.

1. Remove growth media of a 6 well-plate seeded with Vero-TMPRSS2 cells described in step #3 of ‘before you begin’ section and add 2 mL of isolation media per well.
2. Inoculate ~50–150 μL of viral transport media onto the Vero-TMPRSS2 cell monolayer.

   **CRITICAL:** It is essential to include an uninfected control well in the 6 well-plate to monitor for any non-viral induced effects on the cells.

3. Incubate cells at 37°C in 5% CO₂ and monitor daily for potential cytopathic effect (CPE).

   **Note:** Monitor cells up to one week. Usually, CPE is observed within 2–4 days post-inoculation.

4. After ~80%–90% CPE is observed in the infected wells, collect viral supernatants and discard cells.
5. Centrifuge viral supernatants at ~500 × g and 4°C for 10 min to remove any cell debris.
6. Make aliquots.

**Pause point:** Viral supernatants can be stored at −80°C until use.
Plaque purification of SARS-CoV-2 isolates

Timing: 4 days

This step describes how to obtain a clonal viral population from the potential viral quasispecies present in the nasopharyngeal swab and from those that may emerge in cell culture during virus isolation (Figure 1).

7. Perform ten-fold serial dilutions in infection media with the SARS-CoV-2 isolate collected in the step before.
8. Remove the growth media of a 12 well-plate seeded with Vero-TMPRSS2 cells described in step #3 of ‘before you begin’ section.
9. Inoculate 200 μL of the viral dilutions previously prepared onto the confluent Vero-TMPRSS2 monolayer.
10. Move plate to incubator and maintain at 37°C in 5% CO2.

Note: In this step, we do not include undiluted virus. However, it can be used if viral titer is believed to be low.

△ CRITICAL: Rock plates manually every 15 min to ensure an even distribution of the virus in the wells.

11. After 1 h adsorption, discard viral supernatants properly.
12. Add 1 mL of overlay media per well and incubate at 37°C for 3 days. Troubleshooting 1.

Note: Overlay media should be at ~37°C–40°C temperature to not damage the Vero cells monolayer.
13. After three days of incubation, select and mark the viral plaques in the bottom of the plate.

Note: After three days, plaques should be visible by naked eye. Mark multiple plaques (~3–6) from different wells. Select wells corresponding to viral dilutions where plaques are isolated and not contiguous to other plaques to ensure clonal viral populations.

14. Remove the growth media of a new 6 well-plate seeded with Vero-TMPRSS2 cells described in step #3 of ‘before you begin’ section and add 2 mL of infection media per well.
15. Using a 200 μL pipette, pick the selected plaques (~3–6) by going through the agar with the edge of the tip and touching the selected individual plaque.
16. Transfer and resuspend the tip to one of the wells of the 6 well-plate containing infection media.
17. Move plate to incubator and maintain at 37°C in 5% CO2.

\[\triangle CRITICAL: \] Include an uninfected control well in the 6 well-plate to monitor for any non-viral induced effects on the cells.

18. Monitor cells for potential CPE up to a week.
19. After CPE is observed in ~90% of the cells, collect viral supernatants and discard cells properly.
20. Centrifuge viral supernatants at ~500 × g and 4°C for 10 min to remove any cell debris.

\[\triangle Pause point: \] Viral supernatants can be stored at −80°C until use.

Generation of viral stock

\[\circ Timing: \] 3 days

This step allows amplification of the clonal viral population to ensure reproducibility in the following experiments.

21. Inoculate 100 μL of plaque-purified SARS-CoV-2 isolate onto a confluent 175 cm² (~ 25 × 10⁶ cells) flask of Vero-TMPRSS2 cells.
22. Maintain cells in 20 mL of infection media at 37°C in 5% CO₂.
23. After 48–60 h post-infection (when ~80%–90% CPE is observed), collect cell supernatant in falcon tubes and centrifuge for 10 min at 4°C and at ~500 × g. Troubleshooting 2.
24. Make aliquots of 300 μL in cryovials and store at −80°C until use.
25. Sequence viral stocks generated from plaque purification. Troubleshooting 3.

\[\triangle Pause point: \] Viral supernatants can be stored at −80°C until use.

\[\triangle CRITICAL: \] The sequence from the viral stock should be compared with that of the clinical samples generated in previous steps. Therefore, next-generation sequencing should be performed upon collection of nasal swab sample and after generation of viral stock. This is a quality control assay to make sure no mutations take place during amplification of the virus in Vero-TMPRSS2. For further reference see methods (Escalera et al., 2022; Aydillo et al., 2020; Gonzalez-Reiche et al., 2020).

Titration by plaque assay analysis

\[\circ Timing: \] 4 days

Plaque assay analysis allows to calculate and determine the infectious viral particles present in a sample.
26. Perform ten-fold serial dilutions in infection media with the SARS-CoV-2 stock generated in the step before.

27. Remove the growth media of the 12 well-plates seeded with Vero-TMPRSS2 cells described in step #3 of ‘before you begin’ section.

28. Inoculate 200 μL of the viral dilutions onto the confluent Vero-TMPRSS2 monolayer.

29. Move plate to incubator and maintain at 37°C in 5% CO₂.

   **Note**: For this step, no uninfected control is required. Duplicates of each sample should be performed to obtain an accurate titer.

   △ CRITICAL: Rock plates manually every 15 min to ensure an even distribution of the virus in the wells.

30. After 1 h adsorption, remove viral supernatants and discard properly.

31. Add 1 mL of overlay media per well and incubate at 37°C for 3 days. Troubleshooting 1.

   △ CRITICAL: Overlay media needs to be heated until a transparent homogeneous suspension is observed. Excessive heating may result in cell death.

32. After 3 days, fix plates by directly adding 1 mL of 10% formaldehyde per well for 1 h.

   △ CRITICAL: Fixation with 10% formaldehyde is required for inactivation of potential SARS-CoV-2 virus. Decontaminate plates before bringing them out of the BSL-3 facility. The next step in the protocol can be performed in a BSL-2 laboratory.

**Immunostaining**

⊙ **Timing**: 24 h

This step is required to detect infectious virus using specific antibodies and visualize viral plaques for counting and assessment of viral titer.

33. Appropriately, discard 10% formaldehyde in the fume hood.

34. Carefully, remove the overlay from the wells using a spatula.

35. Block the plates for 1 h with shaking using 250 μL of blocking solution per well.

36. After 1 h, remove the blocking solution and add 250 μL per well of mouse anti-SARS-CoV-2 NP antibody (1C7C7) at a dilution of 1:1000 in 1% milk-TBST and incubate for 1 h with shaking and at RT.

37. Wash cells twice with 1× PBS.

38. Add 250 μL per well of goat anti-mouse secondary IgG-HRP antibody at a dilution of 1:5000 in 1% milk-TBST and incubate for 1 h in shaking and at RT.

39. Wash cells twice with 1× PBS.

40. Develop plaques by adding 200 μL of TrueBlue substrate for 15 min in shaking and RT (see Figure 2).

41. Wash plates with water and count plaques.

   **Note**: Count the wells with 10–100 plaques. To determine the viral titer of the initial viral stock (pfu/mL): (average number of plaques/10⁻ⁿ dilution) ×(1 mL/volume used to inoculate dilution (in mL) (Figure 2). We used 1C7C7 antibody for staining, but another anti-SARS-CoV-2 antibody can be used.

   □ **Pause point**: Once the viral titer is determined, the next steps in the protocol can be planned according to schedule.
Infection of Vero-TMPRSS2 cells with SARS-CoV-2 viral isolates

© Timing: 48 h

This step is required to analyze the efficiency in spike protein cleavage of different SARS-CoV-2 variants in the context of in vitro infection and the role and usage of TMPRSS2 proteases by the different viruses.

42. Remove the growth media of the 12 well-plate seeded with Vero-TMPRSS2 cells described in step #3 of ‘before you begin’ section and add 1 mL/well of infection media.
43. Infect cells with the corresponding SARS-CoV-2 isolate at a multiplicity of infection (MOI) of 0.01.

Note: The MOI is the ratio between number of viral particles and infected cells while PFU is a measurement of the quantity of viruses capable of lysing host cells to form a plaque. Therefore, for an MOI of 1, each cell should be infected with one viral particle. In contrast, 1 pfu/mL indicates that 1 mL of the viral preparation will produce a single plaque in a cell monolayer. Since our viral stock is at $1.23 \times 10^6$ pfu/mL (Figure 2), and in this case, we need 3200 pfu/well, we will use 2.6 mL/well of the viral stock.

44. Incubate cells with the virus for 1 h and then, wash them twice with PBS to ensure removal of non-attached virus.
45. Discard the supernatant properly.
46. After infection, maintain the cells in 1 mL/well of infection media until sample collection.

△ CRITICAL: Infections with SARS-CoV-2 viral isolates need to be performed in a BSL-3 facility following biosafety guidelines.

Sample collection and lysis

© Timing: 1 h
Viral supernatants are collected from SARS-CoV-2 infected cells and lysis buffer is added for subsequent antibody detection of viral proteins.

⚠️ CRITICAL: This step is performed in a BSL-3 facility following biosafety guidelines.

**Note:** Prepare fresh lysis buffer before use.

47. Collect ~1 mL of viral supernatants from SARS-CoV-2 infected cells in an Eppendorf tube after 48 h of infection. Troubleshooting 4.
   a. Spin down viral supernatants at ~500 x g for 5 min.
   b. Transfer viral supernatants to a new Eppendorf tube.

**Note:** This step allows clarification of supernatants and ensures removal of cell debris.

   c. Collect 100 μL of clarified supernatant and add 75 μL of lysis buffer.

⚠️ CRITICAL: Decontaminate Eppendorf tubes. The next steps in the protocol can be performed in a BSL-2 laboratory.

48. Boil samples for 10 min at 100°C.
49. Centrifuge samples for 10 min at 4°C and ~20,000 x g.

**Pause point:** Samples can be stored at -20°C until use.

**SDS-PAGE protein electrophoresis**

**Timing:** 3 h

SDS-PAGE protein electrophoresis allows separation of proteins by their molecular weight. In this step, we are interested in separating the cleaved and uncleaved spike proteins together with SARS-CoV-2 nucleocapsid protein for subsequent detection by specific antibodies. The nucleocapsid is used as virus loading control of the viruses tested.

⚠️ CRITICAL: To avoid protein degradation, viral supernatants must be on ice during the preparation of samples for SDS-PAGE.

50. Mix samples with 5x loading buffer to a final concentration of 1x.
51. Boil samples for 10 min at 100°C.
52. Load 15 μL of each sample in a precast 10% TGX gels (15 wells). Additionally, load 10 μL of pre-stained protein ladder.
   a. Run gel at 60 V until samples are concentrated.
   b. Run gel at 120 V for ~ 1 h or until ladder is separated.
53. Transfer gel to polyvinylidene fluoride (PVDF) membranes using BIO-RAD semi-dry transfer system for 3 min.

**Alternatives:** Conventional electroblotting or wet transfer systems can be also used.

**Antibody detection of SARS-CoV-2 spike protein**

**Timing:** 1 day

Uncleaved (full length, 180 kDa) and cleaved (95 kDa) spike proteins are detected using a specific mouse antibody against the S2 spike domain.
54. Fix membranes with 100% methanol for 1 min at room temperature (RT, 15°C–25°C). 
55. Wash membranes with TBST for 5 min in shaking and RT. 
56. Block membranes with 5% non-fat dry milk containing TBST for 1 h in shaking and RT. 
57. Incubate membranes with primary antibody against SARS-CoV-2 Spike S2 Protein diluted 1:3000 in 3% milk TBST overnight (16–18 h) and at 4°C. Troubleshooting 5. 
58. Wash membranes with TBST for 10 min in shaking and RT. Repeat three times. 
59. Incubate membranes with secondary anti-mouse IgG-HRP antibody diluted 1:5000 in 3% milk TBST for 1 h at RT. 
60. Wash membranes with TBST for 10 min in shaking and RT. Repeat three times. 
61. Develop membranes using chemiluminescent substrate. 

**Note:** To achieve best resolution, use SuperSignal West Pico PLUS Chemiluminescent Substrate or similar.

### Antibody detection of SARS-CoV-2 nucleocapsid protein

© Timing: 1 day

SARS-CoV-2 nucleocapsid (50 kDa) protein is detected using a specific rabbit antibody. Detection of nucleocapsid protein is used as loading control and required for subsequent sample normalization. 

**Note:** No stripping buffer is needed. Specific detection is achieved by using antibodies from different animal hosts to detect SARS-CoV-2 spike and nucleocapsid protein. 

62. Wash membranes with TBST for 10 min in shaking and RT. Repeat twice. 
63. Incubate membranes with primary rabbit antibody against SARS-CoV-2 nucleocapsid protein diluted 1:2000 in 3% milk TBST overnight (16–18 h) and at 4°C. 
64. Wash membranes with TBST for 10 min in shaking and RT. Repeat three times. 
65. Incubate membranes with anti-rabbit secondary IgG-HRP antibody diluted 1:3000 in 3% milk TBST for 1 h at RT. 
66. Wash membranes with TBST for 10 min in shaking and RT. Repeat three times. 
67. Develop membranes using chemiluminescent substrate.

### EXPECTED OUTCOMES

Successful SARS-CoV-2 viral isolation from nasopharyngeal swabs is expected when Ct values are approximately ≤ than 25. The titers of viral stocks should reach to at least 10^5 pfu/mL. After Western blot analysis, one clear band corresponding to the cleaved form of the spike protein; or two bands: a band corresponding to cleaved spike form and a second band corresponding to uncleaved spike, should be detected in samples infected with SARS-CoV-2 isolates. In Figure 3, we observe a band corresponding to cleaved S2 spike protein for both Omicron BA.1 variants (one of them encoding an additional spike mutation A701V), Delta and Mu variants. However, two bands were visible in the case of Lambda Variant of Interest (VOI). Similarly, Figure 4 shows the spike protein cleavage from supernatants of Vero E6 and Vero-TMPRSS2-infected cells. A clear S2 cleaved band was detected for the variants isolated from minks (MiA-1 and MiA-2), a human isolate (hNY7) and Gamma Variant of Concern (VOC). However, hNY6 containing a deletion before the furin cleavage site exhibited a strong band corresponding to uncleaved spike, indicating that an intact furin site is required for spike protein processing. When infections were performed in Vero-TMPRSS2 cells, we also observed an efficient spike cleavage in the Gamma, mink and hNY7 variants compared to hNY6. Additionally, we observed S2 cleaved spike in Alpha variant, suggesting the dependance of this VOC for TMPRSS2 proteases. This indicates the importance of studying the spike cleavage of different SARS-CoV-2 variants using multiple cell lines to assess the role and usage of cellular proteases in spike processing. Importantly, all VOCs, VOIs and hNY7 and mink variants used in these
experiments harbor spike mutations such as the S:655Y or P681R polymorphisms, which have been previously described to enhance spike protein cleavage (Escalera et al., 2022; Peacock et al., 2021). Therefore, differences on spike protein cleavage observed in SARS-CoV-2 VOC and VOIs using this protocol could be attributed to presence of different combinations of mutations in the spike protein. Additionally, a band for nucleocapsid protein should be detected in all infected samples (Figures 3 and 4).

QUANTIFICATION AND STATISTICAL ANALYSIS

Western blot quantification was performed using ImageJ (https://imagej.nih.gov/ij/download.html) (see Table 1 related to Figure 3 and, Tables 2 and 3 related to Figure 4). Spike protein levels were normalized to nucleocapsid expression. For this, a normalization lane factor was calculated by dividing the nucleocapsid value in each sample by the highest nucleocapsid protein signal on the blot. Calculation of this factor allows normalization of the experimental intensity values in our experiment. Next, total spike, full length and S2 spike levels were divided by its corresponding normalization lane factor. Finally, percentages were calculated for full length and S2 protein and illustrated using GraphPad Prism (see Figure 5).

LIMITATIONS

This protocol provides a detailed step-by-step method to isolate SARS-CoV-2 human variants from respiratory or mucosal samples (nasopharyngeal swab or saliva) from COVID-19 individuals. However, incorrect storage and transportation of swab samples can affect viral isolation. Nasopharyngeal swabs must be stored at 4°C (not longer than 4 days) or at −80°C. If Ct values are too high, and therefore viral loads are low, no viral isolation is possible. To avoid protein degradation, viral supernatants must be on ice during the preparation of samples for SDS-PAGE. Multiple steps of this protocol need to be performed in BSL-3 level. When generating viral stocks, some SARS-CoV-2 variants may require different timing until collection (for example Omicron may require an extra ~72–84 h post-infection). To detect cleaved spike protein of SARS-CoV-2 variants, an anti-spike S2 monoclonal antibody is used. Mutations in the S2 domain have been shown to be less frequent in newly emerged SARS-CoV-2 variants compared to polymorphisms in the S1 domain, especially in the receptor binding domain (RBD). However, it is possible that new S2 mutations may arise and affect the antibody detection by Western blot.

TROUBLESHOOTING

Problem 1
No plaques were detected in the plaque assay analysis (steps 12 and 31).
Potential solution

Excessive heating of the agar solution may result in cell death. Make sure the overlay medium is at correct temperature (~37°C – 42°C) when added to the cell monolayer.

Problem 2

Not enough CPE was observed after 60 h post-infection when generating the viral stocks (step 23).

Potential solution

Collection after 48–60 h post-infection is an estimation based on the MOI and number of cells we used in this protocol. If some of these parameters change, adjustments should be made. Additionally, some SARS-CoV-2 variants may require different timing until collection, for example Omicron may require an extra ~72–84 h post-infection.

Problem 3

After comparing the sequence from both viral stock and clinical sample, we observe that the viral stock generated after plaque purification has acquired mutations in the spike protein that were not present in the clinical sample. These mutations can have an impact on spike protein cleavage and can emerge when the virus is passage in Vero cells (step 25).

Potential solution

If this is the case, we can sequence initial viral stock generated during isolation of the virus from the nasopharyngeal swab. If there is high abundance of wild type sequence, we can perform another plaque purification and stock generation step to obtain additional isolates from individual plaques. These can be sequenced, and genome confirmed. Once the wild type virus is recovered, comparison between wild type and mutant can be done and effect of the mutations acquired can be determined.
In contrast, and if it is not possible to recover wild type virus from the first passages, one can still characterized the mutant virus alone, but the results need to be interpreted in the context of the mutations.

**Problem 4**
Low SARS-CoV-2 spike and nucleocapsid protein concentration in viral supernatants (step 47).

**Potential solution**
To increase the concentration of protein for successful Western blot detection, different approaches can be used: infect with higher MOI (0.1–1); or infect with lower MOI (0.01–0.001) and collect at later timepoints; or use cell extracts. If using cell extracts, a cell loading control needs to be included.

**Problem 5**
No detection of spike protein of SARS-CoV-2 variants when Western blot is performed due to mutations present in the S2 domain of the spike protein (step 57).

**Potential solution**
The emergence of mutations in the S2 domain has been shown to be less frequent compared to polymorphisms in the S1 spike subunit, especially in the receptor binding domain (RBD). So far, the anti-S2 antibody used for Western blot analysis in this protocol was able to detect all SARS-CoV-2 variants tested (including all VOCs and VOIs). However, new S2 mutations may emerge in future SARS-CoV-2 variants, and this could compromise the detection of spike protein by the S2 antibody used in this protocol. Therefore, additional validated antibodies could be used to detect S2 spike (such as GeneTex, Cat# GTX632604); or anti-S1 antibodies that specifically recognize those SARS-CoV-2 variants.

**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Teresa Aydillo (teresa.aydillo-gomez@mssm.edu).

## Table 2. Western blot quantification of spike protein cleavage from supernatants of Vero-E6-infected cells (raw data and data after normalization are shown), related to Figure 4

| VeroE6 | Peak quantification | Normalization lane factor | Normalized values |
|--------|---------------------|---------------------------|------------------|
|        | FL | S2 | N | Total S | Total FL | S2 | FL % | S2 % |
| Gamma  | 130.61 | 6892.23 | 720.63 | 7022.84 | 0.23 | 31188.24 | 580.02 | 30608.22 | 1.86 | 98.14 |
| Alpha  | 450.26 | 1519.68 | 176.44 | 1969.94 | 0.06 | 35731.97 | 8167.14 | 27564.83 | 22.86 | 77.14 |
| MIA-1  | 457.85 | 8642.23 | 2528.75 | 9100.08 | 0.79 | 11516.70 | 579.44 | 10937.27 | 5.03 | 94.97 |
| MIA-2  | 592.09 | 8501.53 | 3200.28 | 9093.62 | 1.00 | 9093.62 | 592.09 | 8501.53 | 6.51 | 93.49 |
| hNY6   | 5405.63 | 1576.41 | 3003.28 | 6982.03 | 0.94 | 7440.02 | 5760.21 | 1679.81 | 77.42 | 22.58 |
| hNY7   | 368.09 | 6860.41 | 1003.80 | 7228.50 | 0.31 | 23045.70 | 1173.54 | 21872.16 | 5.09 | 94.91 |

## Table 3. Western blot quantification of spike protein cleavage from supernatants of Vero-TMPRSS2-infected cells (Raw data and data after normalization are shown), related to Figure 4.

| Vero-TMPRSS2 | Peak quantification | Normalization lane factor | Normalized values |
|--------------|---------------------|---------------------------|------------------|
|              | FL | S2 | N | Total S | Total FL | S2 | FL % | S2 % |
| Gamma        | 3470.46 | 11414.13 | 3248.11 | 14884.59 | 1.00 | 14884.58 | 3470.45 | 11414.12 | 23.32 | 76.68 |
| Alpha        | 475.34 | 9249.13 | 2818.28 | 9724.47 | 0.87 | 11207.58 | 547.83 | 10659.75 | 4.89 | 95.11 |
| MIA-1        | 71.78 | 5992.89 | 1898.87 | 6064.67 | 0.58 | 10373.91 | 122.78 | 10251.13 | 1.18 | 98.82 |
| MIA-2        | 102.66 | 6721.31 | 3056.87 | 6823.96 | 0.94 | 7250.87 | 109.08 | 7141.79 | 1.50 | 98.50 |
| hNY6         | 3782.34 | 503.58 | 1686.16 | 4285.91 | 0.52 | 8256.09 | 7286.03 | 970.06 | 88.25 | 11.75 |
| hNY7         | 848.04 | 8633.77 | 2135.46 | 9481.81 | 0.66 | 14422.20 | 1289.90 | 13132.29 | 8.94 | 91.06 |
Materials availability
This study did not generate new unique materials.

Data and code availability
This study did not generate unique any datasets or code.

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AUTHOR CONTRIBUTIONS
T.A. and A.G.-S. conceived, designed, and supervised research. T.A. provided training to A.E. A.E. performed experiments. A.E. and T.A. analyzed data and wrote the manuscript. A.G.-S. revised the manuscript and acquired funding.

DECLARATION OF INTERESTS
The A.G.-S. laboratory has received research support from Pfizer, Senhwa Biosciences, Kenall Manufacturing, Avimex, Johnson & Johnson, Dynavax, 7Hills Pharma, Pharmamar, ImmunityBio, Accurius, Nanocomposix, Hexamer, N-fold LLC, Model Medicines, Atea Pharma, and Merck outside of the reported work. A.G.-S. has consulting agreements for the following companies involving cash and/or stock: Vivaldi Biosciences, Contrafect, 7Hills Pharma, Avimex, Vaxalto, Pagoda, Accurius, Esperoxax, Farmak, Applied Biological Laboratories, Pharmamar, Paratus, CureLab Oncology, CureLab Veterinary, Synairgen, and Pfizer outside of the reported work. A.G.-S. has been an invited speaker in meeting events organized by Sequirus, Janssen, and AstraZeneca. A.G.-S. is an inventor on patents and patent applications on the use of antivirals and vaccines for the treatment and prevention of virus infections and cancer, owned by the Icahn School of Medicine at Mount Sinai, New York, outside of the reported work.
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