Aug 03, 2025

# In Vitro Antiviral Screening assay Against Viruses of Pandemic Potential

DOI
https://dx.doi.org/10.17504/protocols.io.5qpvo9jddv4o/v1

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**DOI:** https://dx.doi.org/10.17504/protocols.io.5qpvo9jddv4o/v1

**Protocol Citation:** Nick Lynch 2025. In Vitro Antiviral Screening assay Against Viruses of Pandemic Potential . **protocols.io** https://dx.doi.org/10.17504/protocols.io.5qpvo9jddv4o/v1

**Protocol status:** Working
**We use this protocol and it's working**

**Created:** March 03, 2025

**Last Modified:** August 03, 2025

**Protocol Integer ID:** 124240

**Keywords:** antiviral, cytotoxicity, protease, screening, drug discovery, panel, vitro antiviral screening assay, vitro antiviral screening assay against virus, evaluating antiviral compound efficacy, antiviral compound efficacy, antiviral activity, compounds with antiviral activity, vero 76 cell monolayer, virus inoculation, virus control, test compound, pandemic potential, virus, cell monolayer, compound, reduction assay, known active drug

## Abstract

This protocol details a primary cytopathic effect (CPE) reduction assay for evaluating antiviral compound efficacy. Vero 76 cell monolayers are prepared in 96-well plates and exposed to eight serial half-log10 concentrations of test compounds, along with infected and uninfected controls, and a known active drug. After virus inoculation and incubation until >80% CPE is observed in virus controls, cell viability is quantified using neutral red staining and spectrophotometric analysis at 540 nm. The 50% effective (EC50) and 50% cytotoxic (CC50) concentrations are determined by regression analysis, and the selectivity index (SI50) is calculated to identify compounds with antiviral activity (EC50 <10 µM, SI50 > 5).

## Guidelines

**Compounds need should be pre-weighed into tubes clearly marked with the compound name, exact weight, molecular weight, and Compound number.**

This information can also be included as a table. Starting solutions [M] 10 millimolar (mM) or [M] 3.2 millimolar (mM) solutions if they are stable in solution. If 10 mM is used, half the volume may be sent.

Figure 1. Antiviral Compound Assay Schematic

| | A | B | C | D | E | F | G | H | I | J | K | L | M | N |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Drug [µM] | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | Drug [µM] |
| | 32 | D1 | D1 | D1 V | D1 V | D1 V | C | C | DP V | DP V | DP V | DP | DP | 32 |
| | 10 | D1 | D1 | D1 V | D1 V | D1 V | C | C | DP V | DP V | DP V | DP | DP | 10 |
| | 3.2 | D1 | D1 | D1 V | D1 V | D1 V | C | C | DP V | DP V | DP V | DP | DP | 3.2 |
| | 1.0 | D1 | D1 | D1 V | D1 V | D1 V | Blank | Blank | DP V | DP V | DP V | DP | DP | 1.0 |
| | 0.32 | D1 | D1 | D1 V | D1 V | D1 V | Blank | Blank | DP V | DP V | DP V | DP | DP | 0.32 |
| | 0.1 | D1 | D1 | D1 V | D1 V | D1 V | V | V | DP V | DP V | DP V | DP | DP | 0.1 |
| | 0.032 | D1 | D1 | D1 V | D1 V | D1 V | V | V | DP V | DP V | DP V | DP | DP | 0.032 |
| | 0.01 | D1 | D1 | D1 V | D1 V | D1 V | V | V | DP V | DP V | DP V | DP | DP | 0.01 |

D1 = DRUG #1
DP = POSITIVE CONTROL DRUG
C = Cell controls, cells fed with MEM + 2% FBS and 50 ug/mL gentamicin MEM + 10 U/mL trypsin & 1 ug/mL EDTA for influenza
V = Virus stock (≤100 CCID50 for most viruses)

Table 1. Procedural details for in vitro screening with various viruses. Highlighted viruses are representatives that may be used for a broad-screen of multiple virus groups.

| Virus | Strain | Cell line | Control | Incb. days |
|---|---|---|---|---|
| Heartland | MO-4 | Vero[b] | Ribavirin | 7 |
| Junin | Candid #1 | Vero | Ribavirin | 7-10 |
| La Crosse | Wisconsin 1960 | Vero 76 | Ribavirin | 3 |
| LCMV | Armstrong | Vero[b] | Ribavirin | 7 |
| Maporal | HV97021050 | Vero E6 | T-705 | 7 |
| Oropouche | BE AN 19991 | Vero E6 | Ribavirin | 3 |
| Pichinde | AN 4763 | Vero | Ribavirin | 7-10 |
| Punta Toro | Adames | Vero 76 | Ribavirin | 5 |
| Rift Valley fever | MP-12 | Vero 76 | Ribavirin | 5 |
| SFTSV | HB-29 | Vero[b] | Ribavirin | 7 |
| Tacaribe | TRVL 11573 | Vero | Ribavirin | 7 |
| Alpha human CoV | 229E | Huh7 | EIDD-1931 | 7 |
| Beta human CoV | OC43 | RD | EIDD-1931 | 5 |
| SARS-CoV-1 | Urbani | Vero E6 | Remdesivir | 3 |
| SARS-CoV-2 | WA1/2020 | Vero E6 | EIDD-1931 | 3 |
| MERS CoV | EMC | Vero 76 | Remdesivir | 5 |
| Dengue 2 | New Guinea C | Huh7 | NITD008 | 6 |
| Japanese encephalitis | SA-14 | Vero 76 | NITD008 | 6 |
| Powassan | LB, DTV | BHK-21 | Infergen® | 5 |
| Usutu | TC-508 | Vero 76 | NITD008 | 6 |
| West Nile | Kern 515-WN02 | Vero 76 | NITD008 | 6 |
| Yellow fever | 17D | Huh7 | Infergen® | 5 |
| Zika | MR766 (Uganda) | Vero 76 | NITD008 | 3 |
| Influenza A (H1N1) | A/Michigan/45/2015 | MDCK | Ribavirin | 6 |
| Influenza B | Victoria | MDCK | Ribavirin | 6 |
| Human Parainfluenza | 14702 (PIV3) | MA-104 | Ribavirin | 6 |
| Measles | CC | Vero 76 | 2'-F-2'-deoxyC | 6 |
| Coxsackie | A6 | Vero 76 | Enviroxime | 3 |
| Coxsackie | B3 (HA 201933) | Vero 76 | Enviroxime | 3 |
| Echovirus 11 | Gregory | RD | EIDD-1931 | 3 |
| Echovirus 30 | Bastianni, Frater | RD | EIDD-1931 | 3 |
| Enterovirus-68 | US/MO/14-18947 | RD | Enviroxime | 3 |
| Enterovirus-71 | Tainan/4643/98 | Vero 76 | Pirodavir | 2 |
| Human rhinovirus-14 | 1059 | HeLa-Ohio | Pirodavir | 3 |
| Poliovirus Type 1 | Mahoney (Type 1) | Vero 76 | Enviroxime | 4 |
| Respiratory syncytial | A2 | RD | Ribavirin | 6 |
| Chikungunya | S27 | Vero 76 | EIDD-1931 | 4 |
| EEEV | FL93-939 | Vero 76 | Infergen® | 3 |
| Mayaro | BE AR 505411 | Vero 76 | T-705 | 3 |
| VEEV | TC-83 | Vero 76 | Infergen® | 3 |
| WEEV | California | Vero 76 | Infergen® | 3 |

Table 2. Cell lines that are validated for antiviral testing with the proposed viruses

| Order/Family | Virus | Principal cell line* | Validated alternate cell lines[a, *] |
|---|---|---|---|
| *Bunyavirales* | Heartland | Vero[b] | Huh7[c] |
| | Junin | Vero | BHK-21, BS-C-1 |
| | La Crosse | Vero 76 | Huh7 |
| | Lymphocytic choriomeningitis | Vero[b] | Huh7, A549 |
| | Maporal | Vero E6 | Huh7, A549 |
| | Oropouche | Vero E6 | [a] |
| | Pichinde | Vero | Huh7[c], BS-C-1 |
| | Punta Toro | Vero 76 | Huh7, LLC-MK2 |
| | Rift Valley fever | Vero 76 | Huh7 |
| | SFTSV | Vero[b] | Huh7[c] |
| | Tacaribe | Vero | BS-C-1 |
| *Coronaviridae* | Alpha human coronavirus | Huh7 | HEL 299 |
| | Beta human coronavirus | RD | A549, HEL 299 |
| | SARS-CoV-1 | Vero E6 | Vero 76, Caco2 |
| | SARS-CoV-2 and variants | Vero E6 | A549, ACE2, Vero 76, Caco2 |
| | MERS coronavirus | Vero 76 | HEK[c] |
| *Flaviviridae* | Dengue fever | Huh7 | RD[c], Vero 76 |
| | Japanese encephalitis | Vero 76 | Huh7 |
| | Powassan | BHK-21 | Huh7[c] |
| | Usutu | Vero 76 | Huh7 |
| | West Nile | Vero 76 | Huh7 |
| | Yellow Fever | Huh7 | Vero 76 |
| | ZIKA | Vero 76 | Huh7 |
| *Orthomyxoviridae* | Influenza A | MDCK | MA-104 |
| | Influenza B | MDCK | [a] |
| *Paramyxoviridae* | Human Parainfluenza | MA-104 | [a] |
| | Measles | Vero 76 | Huh7[c], CV-1[c] |
| *Picronaviridae* | Coxsackie A6 | Vero 76 | [a] |
| | Coxsackie B3 | Vero 76 | HeLa, RD[c], HEK, Huh7 |
| | Echovirus 11 | RD | HeLa |
| | Echovirus 30 | RD | HeLa |
| | Enterovirus-68 | RD | A549, HeLa-Ohio |
| | Enterovirus-71 | Vero 76 | RD |
| | Human rhinovirus-14 | HeLa-Ohio | [a] |
| | Poliovirus | Vero 76 | Huh7, A549, HeLa-Ohio, RD |
| *Pneumoviridae* | Respiratory syncytial | RD | MA-104 |

| Order/Family | Virus | Principal cell line* | Validated alternate cell lines[a, *] |
|---|---|---|---|
| *Togaviridae* | Chikungunya | Vero 76 | Huh7, RD |
| | Eastern equine encephalitis | Vero 76 | Huh7[c], RD |
| | Mayaro | Vero 76 | Huh7 |
| | Venezuelan equine encephalitis | Vero 76 | Huh7[c], RD |
| | Western equine encephalitis | Vero 76 | Huh7[c], RD |

[a]Identification and validation of additional cell lines is in process, especially seeking human cell lines.

[b]Dose-response tests are done by VYR assay because this virus does not produce measurable CPE in this cell line.

[c]Alternate cell line validated for virus yield reduction (secondary assay) only due to lack of CPE produced by the virus.

∗Cell origins. Human: Caco2, A549, HeLa, HeLa-Ohio, HEK, HEL 299, HEp2, Huh-7, RD, Calu- 3, dNHBE; Monkey: BS-C-1, CV-1, LLC-MK2, MA-104, Vero, Vero e6, Vero 76; Canine: MDCK;
Hamster: BHK-21

## Materials

**Compound Quantities:**

Minimum amount needed for the number of assays

| | A | B | C |
|---|---|---|---|
| | # Assays | mg Powder | µL of a 3.2 mM  solution* |
| | 1 | 0.5 | 42 |
| | 2 | 0.6 | 74 |
| | 3 | 0.7 | 106 |
| | 4 | 0.8 | 138 |
| | 5 | 0.9 | 170 |
| | 6 | 1.0 | 202 |
| | 7 | 1.1 | 234 |
| | 8 | 1.2 | 266 |
| | 9 | 1.3 | 298 |
| | 10 | 1.4 | 330 |
| | 11 | 1.5 | 346 |
| | 12 | 1.6 | 362 |
| | 13 | 1.7 | 378 |
| | 14 | 1.8 | 394 |
| | 15 | 1.9 | 410 |
| | 16 | 2.1 | 426 |
| | 17 | 2.2 | 442 |
| | 18 | 2.3 | 458 |
| | 19 | 2.4 | 474 |
| | 20 | 2.5 | 490 |
| | 21 | 2.6 | 506 |

## Reduction of virus-induced cytopathic effect (Primary CPE assay)

2h 30m

1 Prepare the confluent or near-confluent cell culture monolayers of Vero 76 cells (or another appropriate cell line) in 96-well disposable microplates the day before testing.

2 Maintain the cells in MEM supplement with 5% FBS.

3 For antiviral assays, use the same medium but with FBS reduced to 2% and supplement with 50 µL gentamicin.

4 Dissolve the compounds in DMSO, saline, or the diluent requested by the submitter. Vortex, heat, and/or sonicate the less soluble compounds and test if they still do not go into solution as colloidal suspensions.

5 Prepare the test compound is at eight serial half-$log_{10}$ concentrations, usually [M] 32 micromolar (µM), [M] 10 micromolar (µM), [M] 3.2 micromolar (µM), [M] 1.0 micromolar (µM), [M] 0.32 micromolar (µM), [M] 0.1 micromolar (µM), [M] 0.032 micromolar (µM) and [M] 0.01 micromolar (µM) (or per sponsor preference).

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Figure 1. Antiviral Compound Assay Schematic

| Drug [µM] | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | Drug [µM] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 32 | D1 | D1 | D1 V | D1 V | D1 V | C | C | DP V | DP V | DP V | DP | DP | 32 |
| 10 | D1 | D1 | D1 V | D1 V | D1 V | C | C | DP V | DP V | DP V | DP | DP | 10 |
| 3.2 | D1 | D1 | D1 V | D1 V | D1 V | C | C | DP V | DP V | DP V | DP | DP | 3.2 |
| 1.0 | D1 | D1 | D1 V | D1 V | D1 V | Blank | Blank | DP V | DP V | DP V | DP | DP | 1.0 |
| 0.32 | D1 | D1 | D1 V | D1 V | D1 V | Blank | Blank | DP V | DP V | DP V | DP | DP | 0.32 |
| 0.1 | D1 | D1 | D1 V | D1 V | D1 V | V | V | DP V | DP V | DP V | DP | DP | 0.1 |
| 0.032 | D1 | D1 | D1 V | D1 V | D1 V | V | V | DP V | DP V | DP V | DP | DP | 0.032 |
| 0.01 | D1 | D1 | D1 V | D1 V | D1 V | V | V | DP V | DP V | DP V | DP | DP | 0.01 |

D1 = DRUG #1
DP = POSITIVE CONTROL DRUG
C = Cell controls, cells fed with MEM + 2% FBS and 50 ug/mL gentamicin
MEM + 10 U/mL trypsin & 1 ug/mL EDTA for influenza
V = Virus stock (≤100 $CCID_{50}$ for most viruses)

7 Use the lower concentrations when insufficient compound is supplied or when a lower starting concentration is requested.

8 Use the five microwells per dilution: three for infected cultures and two for uninfected toxicity cultures.

9 Controls for the experiment consist of six microwells that are infected and not treated (virus controls) and six that are untreated and uninfected (cell controls) on every plate.

10 Test a known active drug in parallel as a positive control drug using the same method as is applied for test compounds. Test the positive control with every test run.

11 On the testing day, remove the growth media from the cells and the test compound is applied in 0.1 ml volume to wells at 2X concentration.

12 Virus, normally at a titer that will cause >80% CPE (usually an MOI <0.003), in 0.1 mL volume is added to the wells designated for virus infection.

13 Place the medium devoid of virus in toxicity control wells and cell control wells.

14 Incubate the plates at 37 °C with 5% $CO_2$ until marked CPE (>80% CPE for most virus strains) is observed in virus control wells.

15 Then stain the plates with 0.011% neutral red for approximately 02:00:00 at 37 °C in a 5% $CO_2$ incubator. 2h

16 Remove the neutral red medium by complete aspiration, and may rinse the cells 1X with phosphate-buffered solution (PBS) to remove the residual dye.

17 Remove the PBS completely, and elute the incorporated neutral red with 50% Sorensen's citrate buffer/50% ethanol for at least 00:30:00. 30m

18 Neutral red dye penetrates into living cells, thus, the more intense the red color, the larger the number of viable cells present in the wells.

19 Quantify the dye content in each well using a spectrophotometer at 540 nm wavelength.

20 Convert the dye content in each set of wells to a percentage of dye present in untreated control wells using a Microsoft Excel-based spreadsheet and normalized based on the virus control.

21 Then calculate the 50% effective ($EC_{50}$, virus-inhibitory) concentrations and 50% cytotoxic ($CC_{50}$, cell-inhibitory) concentrations by regression analysis.

22 Divide the quotient of $CC_{50}$ by $EC_{50}$ gives the 50% selectivity index ($SI_{50}$) value. Compounds showing $EC_{50}$ < [M] 10 micromolar (µM) and SI50 values > 5 are considered minimally active.

## Reduction of virus yield (VYR assay) 2h 30m

23 Test the active compounds further in a confirmatory VYR assay. This assay is run for compounds that have an $EC_{50}$ < [M] 10 micromolar (µM) and $SI_{50}$ ≥ 5.

24 After sufficient virus replication occurs (generally 3 days for many viruses), a sample of supernatant is taken from each infected well (replicate wells are pooled) and held frozen

at -80 °C for later virus titer determination.

25 After maximum CPE is observed, stain the viable plates with neutral red dye. Quantify the incorporated dye content as described above to generate the $EC_{50}$ and $CC_{50}$ values.

26 The VYR test is a direct determination of how much test compound is required to inhibit 90% virus replication.

27 Titrate the virus yielded in the presence of the test compound and compare to virus titers from the untreated virus controls.

28 Perform the titration of the viral samples (collected as described in the paragraph above) by endpoint dilution (Reed and Muench).

29 Make the serial 10-fold dilutions of supernatant and plate into 4 replicate wells containing fresh cell monolayers of Vero 76 cells.

30 Incubate the plates, and score the cells for the presence or absence of the virus after distinct CPE is observed, and calculate the $CCID_{50}$ using the Reed-Muench method (24).

31 Calculate the 90% (one $log_{10}$) effective concentration ($EC_{90}$) by regression analysis by plotting the $log_{10}$ of the inhibitor concentration versus log10 of the virus produced at each concentration. Dividing $EC_{90}$ by the $CC_{50}$ gives the $SI_{90}$ value for this test.

## Summary of virus panel

32 Antiviral Summary

**Table 1.** Procedural details for in vitro screening with various viruses. Highlighted viruses are representatives that may be used for a broad-screen of multiple virus groups.

| Virus | Strain | Cell line | Control | Incb. days |
|---|---|---|---|---|
| Heartland | MO-4 | Vero[b] | Ribavirin | 7 |
| Junin | Candid #1 | Vero | Ribavirin | 7-10 |
| La Crosse | Wisconsin 1960 | Vero 76 | Ribavirin | 3 |
| LCMV | Armstrong | Vero[b] | Ribavirin | 7 |
| Maporal | HV97021050 | Vero E6 | T-705 | 7 |
| Oropouche | BE AN 19991 | Vero E6 | Ribavirin | 3 |
| Pichinde | AN 4763 | Vero | Ribavirin | 7-10 |
| Punta Toro | Adames | Vero 76 | Ribavirin | 5 |
| Rift Valley fever | MP-12 | Vero 76 | Ribavirin | 5 |
| SFTSV | HB-29 | Vero[b] | Ribavirin | 7 |
| Tacaribe | TRVL 11573 | Vero | Ribavirin | 7 |
| Alpha human CoV | 229E | Huh7 | EIDD-1931 | 7 |
| Beta human CoV | OC43 | RD | EIDD-1931 | 5 |
| SARS-CoV-1 | Urbani | Vero E6 | Remdesivir | 3 |
| SARS-CoV-2 | WA1/2020 | Vero E6 | EIDD-1931 | 3 |
| MERS CoV | EMC | Vero 76 | Remdesivir | 5 |
| Dengue 2 | New Guinea C | Huh7 | NITD008 | 6 |
| Japanese encephalitis | SA-14 | Vero 76 | NITD008 | 6 |
| Powassan | LB, DTV | BHK-21 | Infergen® | 5 |
| Usutu | TC-508 | Vero 76 | NITD008 | 6 |
| West Nile | Kern 515-WN02 | Vero 76 | NITD008 | 6 |
| Yellow fever | 17D | Huh7 | Infergen® | 5 |
| Zika | MR766 (Uganda) | Vero 76 | NITD008 | 3 |
| Influenza A (H1N1) | A/Michigan/45/2015 | MDCK | Ribavirin | 6 |
| Influenza B | Victoria | MDCK | Ribavirin | 6 |
| Human Parainfluenza | 14702 (PIV3) | MA-104 | Ribavirin | 6 |
| Measles | CC | Vero 76 | 2'-F-2'-deoxyC | 6 |
| Coxsackie | A6 | Vero 76 | Enviroxime | 3 |
| Coxsackie | B3 (HA 201933) | Vero 76 | Enviroxime | 3 |
| Echovirus 11 | Gregory | RD | EIDD-1931 | 3 |
| Echovirus 30 | Bastianni, Frater | RD | EIDD-1931 | 3 |
| Enterovirus-68 | US/MO/14-18947 | RD | Enviroxime | 3 |
| Enterovirus-71 | Tainan/4643/98 | Vero 76 | Pirodavir | 2 |
| Human rhinovirus-14 | 1059 | HeLa-Ohio | Pirodavir | 3 |
| Poliovirus Type 1 | Mahoney (Type 1) | Vero 76 | Enviroxime | 4 |
| Respiratory syncytial | A2 | RD | Ribavirin | 6 |
| Chikungunya | S27 | Vero 76 | EIDD-1931 | 4 |
| EEEV | FL93-939 | Vero 76 | Infergen® | 3 |
| Mayaro | BE AR 505411 | Vero 76 | T-705 | 3 |
| VEEV | TC-83 | Vero 76 | Infergen® | 3 |
| WEEV | California | Vero 76 | Infergen® | 3 |

## Protocol references

Reed, L.J., and H. Muench. "A Simple Method of Estimating Fifty Percent Endpoints." Am J Hyg 27 (1938): 493-98. Print.

## Acknowledgements

We thank Institute for Antiviral Research Utah State University for sharing the protocol of this assay