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Live Virus EV-D68 - RD - Antiviral Screening Assay

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We use this protocol and it's working

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Disclaimer

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Abstract

The following is a protocol for live virus antiviral screening against EV-D68 in vitro using Rhabdomyosarcoma (RD) cells. RD Cells are prophylactically treated with antiviral candidates that have been serially diluted to investigate activity, via IC50 values generated via analysis of immunofluorescent staining, against EV-D68.
You may opt to include PgP inhibitor in the assay by adding 2uM into the infection media.
Each live virus screening assay is accompanied by a matching cytotoxicity screening assay in uninfected cells to investigate drug toxicity.

Materials

1. D300e
   - D300e program on computer
   - D300e machine
   - D4 cassettes
   - T8 cassettes
2. Media
   - Fresh DMSO
   - Infection media:
     a) 500 mL DMEM
     b) 10 mL FBS for 2% FBS
     c) 5 mL P/S
     d) 5 mL NEAA
     e) 5 mL HEPES
3. Compound
   - Stock concentration
   - Working concentration
   - ***Rupintrivir as reference compound***
4. Materials
   - 1× 96-well flat bottom Greiner plate (treated with Poly L-Lysine, black)
   - 1× 96-well flat bottom Greiner plate (treated with Poly L-Lysine, clear)
   - Deep 96-well plate
   - Reservoirs
   - Multichannel Pipette
   - Automatic multichannel pipette
5. Cells
   - RD cells (passage should be low ~15)
6. Virus
   - EVD68 MO/14 virus stock 20180911 EV-D68 Virus stock (MO/14-18347) 20180911 (Titer: 6.25E5 PFU/mL)
   - EVA71 Strain H LLCMK2 7DPI 5/16/22 (Titer: 7.5E5 PFU/mL)

Protocol materials

- Corning® Fetal Bovine Serum **Corning Catalog #**35-010-CV
- HEPES (1M) **Gibco - Thermo Fisher Scientific Catalog #**15630080
- MEM Non-Essential Amino Acids Solution (100X) **Gibco - Thermo Fisher Scientific Catalog #**11140050
- Corning® 500 mL DMEM (Dulbecco's Modified Eagle's Medium) **Corning Catalog #**10-017-CV
- Penicillin-Streptomycin (10,000 U/mL) **Gibco - Thermo Fisher Scientific Catalog #**15140122
- Dimethyl sulfoxide 100mL **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D2650-100ML
- Enterovirus D68 VP1 antibody **Genetex Catalog #**GTX132313
- DAPI and Hoechst Nucleic Acid Stains **Invitrogen - Thermo Fisher Catalog #**H1399
- Goat anti-Rabbit IgG (H L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488 **Invitrogen Catalog #**A-11034
- Bovine serum albumin (BSA) Gemini 700-101P **Gemini Bio-Products Catalog #**700-101P
- Triton X-100 **Fisher Scientific**
- 37% Formaldehyde **Fisher Scientific Catalog #**F79-1
- Phosphate-Buffered Saline, 1X without calcium and magnesium, PH 7.4 ± 0.1 **Corning Catalog #**21-040-CV
- MTT Reagent A **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**CT01-5
- Isopropanol **Fisher Scientific Catalog #**BP2618500
- Corning® 96 well plates **Corning Catalog #**CLS9102
- Clear 96-well flat-bottom microplate **Corning Catalog #**353072
- Falcon® 175cm² Rectangular Straight Neck Cell Culture Flask with Vented Cap **Corning Catalog #**353112
- Corning® Costar® reagent reservoirs **Corning Catalog #**CLS4873
- 2.0ml 96 Well Deep Well Plates Square Well **NEST Biotechnology Catalog #**503001/503501

Troubleshooting

Safety warnings

Please be sure to wear proper Personal Protective Equipment (PPE) while performing this experiment.

Before start

Always wear appropriate PPE for this protocol
Refer to Material Safety Data Sheets for additional safety and handling information.

Summary

1 RD cells (2k per well) were seeded in 96 Well Black/Clear Bottom Plates and incubated overnight. On the following day, cell density was determined using Trypan Blue. Infection media containing DMSO was prepared, and deep 96-well plates were filled with 800 µL per well, except for column 12. Diluted compounds were added to the designated wells. After a 2.5-hour incubation at 37°C with 5% CO2, the test plate was infected with EV-D68 (strain US/MO/14-18949, ATCC/BEI Resources) at an MOI of 1. After 24 hours of infection, media was aspirated, and cells were fixed with 4% Formaldehyde + PBS. Following fixation, formaldehyde was carefully removed and the monolayers were washed once with PBS. PBS was discarded, and cells were incubated with 0.1% Triton X-100 for 20 minutes. The monolayer was washed three times with PBS. Cells were then incubated with 0.3% BSA/PBS for 30 minutes. The primary antibody, Anti-Enterovirus D68 VP1 (GTX132313), was diluted in 0.3% BSA/PBS and added to the wells. Following a 1-hour incubation, cells were washed three times with PBS. The secondary antibody, goat antirabbit IgG (H+L) Alexa Fluor 488 (ab150077), diluted in 0.3% BSA/PBS, was added to the cells and incubated for an additional hour, protected from light. The monolayer was washed three times with PBS and counterstained with DAPI. The final PBS wash was left on the cells for imaging.

Imaging was performed using the Cytation 1 system, Viral infection and cell viability were assessed using fluorescence imaging to quantify Alexa Fluor 488 and DAPI signals. Images were analyzed using Gen5 software to calculate infection rates and determine antiviral
efficacy.

Rupintrivir was used as a positive antiviral control. RD cell cytotoxicity was determined by MTT assays on uninfected cells.

Materials

2 **Plates:**

1. 96- Well Deep Well plate
   2.0ml 96 Well Deep Well Plates Square Well **NEST Biotechnology Catalog #**503001/503501
2. Black 96-well Flat Bottom plate with Clear Wells
   Corning® 96 well plates **Corning Catalog #**CLS9102
3. Clear 96-well Flat Bottom plate
   Clear 96-well flat-bottom microplate **Corning Catalog #**353072

Flask:

Falcon® 175cm² Rectangular Straight Neck Cell Culture Flask with Vented Cap **Corning Catalog #**353112

Reservoirs:

Corning® Costar® reagent reservoirs **Corning Catalog #**CLS4873

3 **Media**:

3.1 **Cell Culture Media:**

500 mL

Corning® 500 mL DMEM (Dulbecco's Modified Eagle's Medium) **Corning Catalog #**10-017-CV

1. 50 mL Corning® Fetal Bovine Serum **Corning Catalog #**35-010-CV
2. 5 mL

   Penicillin-Streptomycin (10,000 U/mL) **Gibco - Thermo Fisher Scientific Catalog #**15140122
3. 5 mL HEPES (1M) **Gibco - Thermo Fisher Scientific Catalog #**15630080

   HEPES (1M) **Gibco - Thermo Fisher Scientific Catalog #**15630080

3.2 **Infection Media:**

1. 500 mL

   Corning® 500 mL DMEM (Dulbecco's Modified Eagle's Medium) **Corning Catalog #**10-017-CV
2. 10 mL Corning® Fetal Bovine Serum **Corning Catalog #**35-010-CV
3. 5 mL

   Penicillin-Streptomycin (10,000 U/mL) **Gibco - Thermo Fisher Scientific Catalog #**15140122
4. 5 mL

   MEM Non-Essential Amino Acids Solution (100X) **Gibco - Thermo Fisher Scientific Catalog #**11140050
5. 5 mL HEPES (1M) **Gibco - Thermo Fisher Scientific Catalog #**15630080

   HEPES (1M) **Gibco - Thermo Fisher Scientific Catalog #**15630080

3.3 **DMSO + Infection Media:**

1. 50 mL of Infection Media (2.2)

2. 150 µL

Dimethyl sulfoxide 100mL **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D2650-100ML

4 **Immunofluorescence Staining:**

4.1 **Antibodies:**

1. Enterovirus D68 VP1 antibody **Genetex Catalog #**GTX132313
2. DAPI and Hoechst Nucleic Acid Stains **Invitrogen - Thermo Fisher Catalog #**H1399
3. Goat anti-Rabbit IgG (H L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488 **Invitrogen Catalog #**A-11034

4.2 **Solutions:**

1. 0.3%

Bovine serum albumin (BSA) Gemini 700-101P **Gemini Bio-Products Catalog #**700-101P

2. 0.1% Triton X-100 **Fisher Scientific**
3. 4% 37% Formaldehyde **Fisher Scientific Catalog #**F79-1
4. Phosphate-Buffered Saline, 1X without calcium and magnesium, PH 7.4 ± 0.1 **Corning Catalog #**21-040-CV

5 **Cytotoxicity Testing:**

1. MTT Reagent A **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**CT01-5
2. Isopropanol **Fisher Scientific Catalog #**BP2618500

6 **Tecan D300e:**

Equipment

Tecan D300e digital dispenser

NAME

Tecan

BRAND

30100152

SKU

Equipment

T8 Dispensehead Cassettes

NAME

Tecan

BRAND

30097370

SKU

7 **Cytation 1:**

Equipment

Agilent BioTek Cytation 1 Cell Imaging Multi-Mode Reader NAME

Aligent BioTek BRAND

BTCYT1FAV SKU

Seeding (Day 1)

8

1. 24 hours prior to treatment, using 96-well plates, seed 2,000 Rhabdomyosarcoma (RD) cells per well (**Figure 1.**) using Cell Culture Media (2.1).
2. Place seeded cells in incubator at 37 °C in 5 % overnight.

Figure 1.

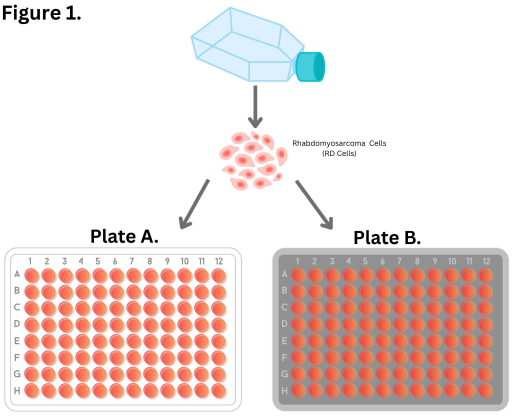


Figure 1. Cell Seeding Layout. Plate A. Clear flat bottom 96- well plate. **Plate B.** Black flat bottom 96-well plate with clear wells. Both plates consist of roughly 2,000 Rhabdomyosarcoma (RD) cells per well 24 hours before treatment.

Figure 1. Cell Seeding Layout. Plate A. Clear flat bottom 96- well plate. **Plate B.** Black flat bottom 96-well plate with clear wells. Both plates consist of roughly 2,000 Rhabdomyosarcoma (RD) cells per well 24 hours before treatment.

8.1 Two types of flat bottom 96-well plates are used: Clear 96-well Flat Bottom plates, and Black 96-well Flat Bottom plates with Clear Wells. Each are paired for the assay. One plate is used for antiviral screening and the other for cytotoxicity screening.

8.2 Three compounds in three replicates are used per each plate pair (**Figure 2**.)

Figure 2.

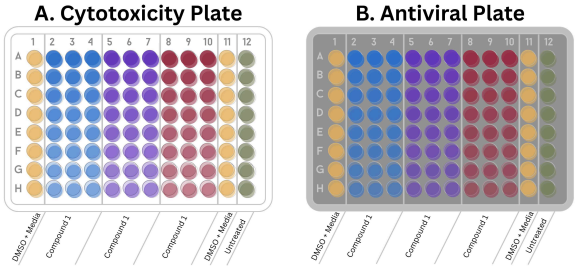


Figure 2. Layout of plates for antiviral and cytotoxicity screening. Plate A. Clear flat bottom 96- well plate used for Cytotoxicity testing of compounds. **Plate B.** Black flat bottom 96-well plate with clear wells used for Antiviral testing of compounds. Both consist of roughly 2,000 Rhabdomyosarcoma (RD) cells per well 24 hours prior to treatment.

Figure 2. Layout of plates for antiviral and cytotoxicity screening. Plate A. Clear flat bottom 96- well plate used for Cytotoxicity testing of compounds. **Plate B.** Black flat bottom 96-well plate with clear wells used for Antiviral testing of compounds. Both consist of roughly 2,000 Rhabdomyosarcoma (RD) cells per well 24 hours prior to treatment.

Compound Dilution Preparation (Day 2)

9 Under the Biosafety Cabinet, use a Deep 96-well plate to prepare the compound dilutions for treatment. One compound will be assigned to one column in the deep 96-well plate (**Figure 3.**)

9.1
1. Pipette 1000 µL of Infection Media (**2.2**) per well for row A (**Figure 3.**)
2. Pipette 1000 µL of DMSO + Infection Media mixture (**2.3**) into the remaining wells (rows B-H) (**Figure 3.**)

Now you do NOT have to normalize the plates to DMSO when using the Tecan D300e digital dispenser.

Figure 3.

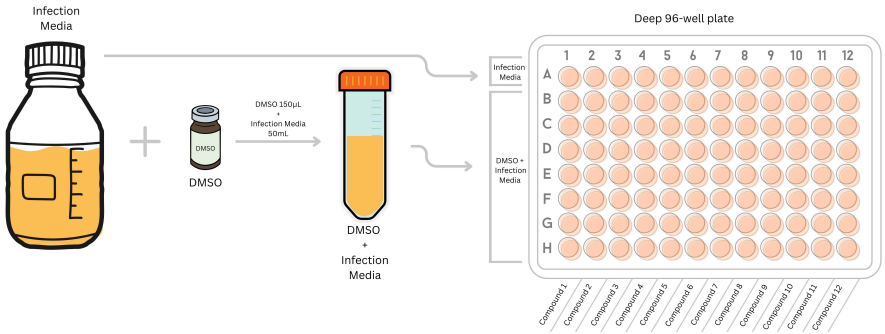


Figure 3. Deep 96-well plate Layout with DMSO + Infection Media. Row A wells containing 1000µL of Infection Media, Row B-H wells containing 1000µL Infection Medica with DMSO.

Figure 3. Deep 96-well plate Layout with DMSO + Infection Media. Row A wells containing 1000µL of Infection Media, Row B-H wells containing 1000µL Infection Medica with DMSO.

Compound Dilutions Using the Tecan D300e Digital Dispenser

10 **Preparing the Compounds:**

1. Thaw the compounds to be tested always including Rupintrivir as a control and organize the samples according to the compound list. Ensure that the order of the compounds matches the list exactly.
2. Use a USB and copy and paste the list of compounds to be tested onto it.

11 **Preparing the Tecan D300e Digital Dispenser:**

11.1 Once samples are thawed, turn on the hood blower and the Tecan D300e Digital Dispenser (**5**). Power on the connecting computer and open the Tecan D300e software.

11.2 Plug in your USB with the list of compounds saved on it. Copy the list exactly and paste the copied list into the fluids section of the Tecan D300e software page. All of the compounds should appear as their own "tabs" on the left-hand tab of the page.

12 Click on "Quick Plate".

12.1 Under "Plate" Tab:

1. Plate Type: 96 Well
2. Additional Volume: 1000 µL
3. DMSO limit (%): 1

Click "Next"

12.2 Under "Titration" Tab:

1. Titration Levels: 8
2. Specify titration using: Highest concentration
3. Highest Concentration: (Highest desired Concentration) x (1.5) = input concentration

   i.e. : ([M] 100 micromolar (µM)) x (1.5)= [M] 150 micromolar (µM)

   All compounds as of 2025 should have a starting concentration of [M] 100 micromolar (µM) *Rupintrivir should have a starting concentration of* [M] 1 micromolar (µM)
4. Distribution: 1:3 (33%)
5. Replicates per level: 1
6. Order: High to Low

Click "Next"

12.3 Under "Layout" Tab:

1. Layout: Layout by columns
2. Interleave fluids: Do not interleave
3. Choose the third display option

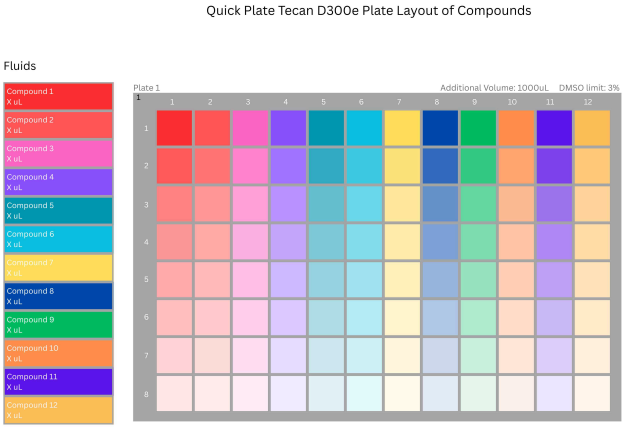
Click "Next"

12.4 Under "Summary" Tab:

Click "Finish"

12.5 All of the compounds listed under Fluids should now be seen in the Plate Layout (**Figure 4.**).

Figure 4.



12.6 Click "Run" at the top left side of the software page.

13 **Diluting the Deep 96-well Plate:**
After preparing the protocol for the D300e program, take your prepared deep 96-well plate and place it on the plate holder of the Tecan D300e machine.

13.1 Place a T8 cassette (**5**) (8x well cassette on top of the D300e machine) and place on the cassette holder.

13.2 Click “loaded”.

13.3 Add in indicated compound amounts into each well of the cassette (1 compound/cassette well).

13.4 Repeat filling in the remaining compounds until finished.

13.5 Click “all filled” and allow the machine to add and dilute the compounds into the deep 96-well plate.

14 Once all compounds are added and properly diluted into the deep 96-well plate(s), make sure to cover the deep well plates to maintain sterility.

Treating and infecting the cells

15 1. Retrieve cell plates from the incubator.
2. Aspirate media from cells of columns 1 and 11 for plate B and plate C (**Figure 5.**).
3. Add in 100 µL of diluted compounds from the deep 96-well plate. Each compound should be added around the same time for both plates.

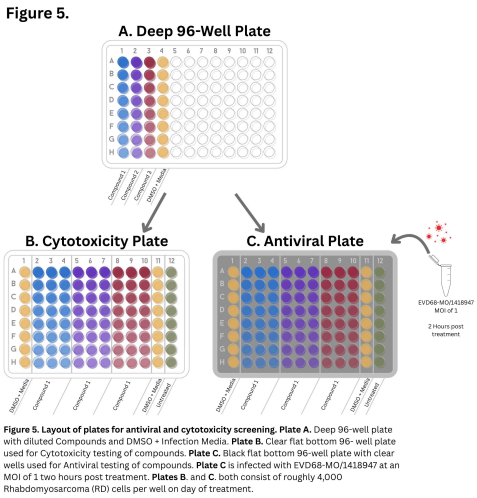


Figure 5. Layout of plates for antiviral and cytotoxicity screening. Plate A. Deep 96-well plate with diluted Compounds and DMSO + Infection Media. **Plate B.** Clear flat bottom 96-well plate used for Cytotoxicity testing of compounds. **Plate C.** Black flat bottom 96-well plate with clear wells used for Antiviral testing of compounds. **Plate C** is infected with EVD68-MO/1418947 at an MOI of 1 two hours post treatment. **Plates B**. and **C**. both consist of roughly 4,000 Rhabdomyosarcoma (RD) cells per well on day of treatment.

16 Repeat steps until all the diluted compound is added to the cells.
The 12th column is used for untreated cells - you do not need to aspirate this column.

17 1. Place cells back in incubator at 37 °C in 5 % for roughly 2.5 hours, then infect plate(s) C (Antiviral Plates) with 50 µL of EVD68-MO/1418947 in Infection Media at an MOI of 1 two hours post treatment.
2. Place plates back in incubator at 37 °C in 5 %

Cytotoxicity Screening and Antiviral Screening Fixing

18 **Cytotoxicity Testing and Fixing**: Perform an MTT for plate(s) B around the same time plate(s) C are fixed.

19 **Cytotoxicity Testing and Fixing**: Perform an MTT for plate(s) B around the same time plate(s) C are fixed.

1. **Cytotoxicity Testing:** MTT Reagent A powder is dissolved in 10mL of 1x PBS for a stock solution. Store the working stock at 4 °C
2. Before you begin, prepare a working stock of MTT Reagent A by diluting the working stock 1:10 in 1x PBS (*i.e.* 1mL stock + 9mL PBS). This is light sensitive, perform with hood light turned off.

Cytotoxicity Screening and Antiviral Screening Fixing

19.1 Gently aspirate off media from cells taking care not to disturb them.

19.2 Add 50uL MTT reagent A per well (GENTLY)

19.3 Incubate treated cells in incubator at 37 °C in 5 % for 3 hours. Cells should appear dark/purple as crystals form in the cells (either by eye or under microscope)

19.4
1. Solubilize with 150 µL 100% Isopropanol at Room temperature for 40 minutes. Make sure to pipette up and down to dissolve crystals/break membranes when adding the Isopropanol. Remember this is light sensitive, wrap in foil to ensure accurate read out.
2. Make sure crystals are fully dissolved.

20 **Cytation 1 Read out for Cytoxicity Screening**: (**23**)

21 **Fixing**: Roughly 24 hours after infecting, aspirate the compound and virus from Plate C (Use a multichannel pipette and Quatricide to discard media waste) and add in 100-150 µL of 4% Formaldehyde + PBS/well to fix the cells for at least 20 minutes. Leave at Room temperature .

Immunostaining (**21**)

Immunostaining (Day 3/4)

22 **Immunostaining**: Immunostain the fixed Plate(s) C.

22.1 **Day 1:**

1. Carefully remove the formaldehyde fixative and wash the monolayers once with PBS
2. Discard the PBS and add 100 µL of 0.1% Triton X-100 (**3.2**) to each well.
3. Incubate the cells in 0.1% Triton X-100 for 20 minutes at Room temperature
4. Carefully wash the monolayer 3x with 1x PBS
5. Discard wash and incubate to block with 0.3% BSA/PBS for an hour (**3.2).**
6. Dilute the primary antibody EVD68 VPI Rabbit (**3.1**) at a 1:800 dilation in 0.3% BSA/PBS, add 50uL per well. Incubate for at least 2 hours Room temperature or preferably overnight at 4 °C

Day 2:

At this point, be careful with light as the assay will be light sensitive

1. Carefully wash the monolayer 3x with 1x PBS
2. Discard wash and dilute the secondary antibody goat anti-Rabbit FITC (alexa- flour) (**3.1**) at a 1:2000 dilation + DAPI in 0.3% BSA/PBS, add 50uL per well and incubate for at least 1 hour at Room temperature .
3. Carefully wash the monolayer 3x with 1x PBS
4. Add 100 µL of fresh 1x PBS to each well for read out.
5. **Cytation 1 Read out for Antiviral Screening: (24)**

Cytation 1 Read out (Day 4/5)

23 **Cytation 1:** Read out with Cytation 1 machine.

24 **Cytation 1, Cytotoxicity Screening:** This is done the same day the Cytotoxicity assay begins. Read absorbance of Plate(s) B using the Cytation 1 machine.

24.1

1. Power on the Cytation 1 Imaging Reader and the computer attached to it.
2. Open up the Gen 5 3.15 Software on the computer
3. Read absorbance at 630 and 570nm of Plate(s) B using the Cytation 1 machine.

25 **Cytation 1, Antiviral Screening:** Read GFP and DAPI stained cells of Plate(s) C using the Cytation 1 machine.

25.1
1. Power on the Cytation 1 Imaging Reader and the computer attached to it.
2. Open up the Gen 5 3.15 Software on the computer
3. Read each individually stained GFP and DAPI stained cell for each well using the untreated as a control for GFP detection of Plate(s) C using the Cytation 1 machine.
4. Data Reduction "Ratio": DS1S Cell Count 2 (GFP) / DS2S Cell Count 1 (DAPI). Set factor to 100 and label as a % Cell Count.
5. "Normalize" % Cell Count to DMSO and express as a ratio.

CDD Formatting Information

26 Export all of the processed data from the Cytation 1 Machine to excel using software Add-ins to convert all information to Collaborative Drug Discovery CDD vault formatting.

27 **Cytotoxicity Screening CDD Format:**
Plate ID: EDRD#
Well: Filled
Well ID: Compound name, DMSO, Untreated, Rupintrivir
Name: ASAP#
Concentration/ Dilution: Filled
570: Replicates
630: Replicates
630/570: Replicates

28 **Antiviral Screening CDD Format:**
Plate ID: EDRD#
Well: Filled
Well ID: Compound name, DMSO, Untreated, Rupintrivir
Name: ASAP#
Concentration/ Dilution: Filled
Cell Count 2: Replicates
Cell Count 1: Replicates
% Cell Count: Replicates