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Antiviral In Vitro Screening Using SARS-CoV-2 and MERS-CoV - Cytotoxicity of Drugs

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We use this protocol and it's working

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Disclaimer

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Abstract

The protocol evaluates compounds to determine their antiviral activity against coronaviruses. The antiviral activity is evaluated based on the 50% inhibitory concentration (IC50) and the 50% effective concentration (EC50). Two cell lines can be used for these assays (A549+ACE2+TMP (Invivogen) or VeroE6/TMPRSS2 cells (XenoTech)). The multiplicity of infection (MOI) will depend on the virus stock titer. The incubation time can vary if using a reporter virus. The assay can be performed in 96-well plates (medium throughput screening) or 384-well plates (high throughput screening).

Materials

DMEM (ATCC® 30-2002),
Trypsin (TrypLE 12604039 Thermofisher),
FBS (C788U22 Thermo Scientific),
10% Neutral buffered formalin (Fisher Scientific Epredia™ Formal-Fix™ 6764240),
Hydrogen peroxide disinfectant (Peroxigard™),
Breathe-Easy sealing membrane (Diversified Biotech),
384 well white plate, clear bottom (FisherSci 07-201-013),
384 well black plates, clear bottom (FisherSci 07-201-442),
96 well white plates, clear bottom (VWR 82050-758),
96 well black plates, clear bottom (Sigma CLS3603),
CellTiter Glo (Promega G9243),
Nano Glo Luciferase Assay System (Promega N1130),
Antimycotic / Antibiotic (ThermoFisher 15240-062),
Tecan D300e Digital dispenser,
Biotek EL-406 washer dispenser,
Integra Biosciences Viaflo 96 / 384 electronic dispenser,
Tecan Infinite 200 PRO plate reader, EVOs Inverted phase contrast microscope.

Safety warnings

Always wear appropriate PPE for this protocol
Samples contain BSL-3 level high risk agents (viral) with infectious aerosol transmission potential.

Before start

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

Purpose

1 The protocol includes the steps for the evaluation of drugs to determine their antiviral activity against
coronaviruses. The antiviral activity is evaluated based on the 50 % inhibitory concentration (IC50) and
the 50% effective concentration (EC50).
Two cell lines can be used for these assays (A549+ACE2+TMP (Invivogen) or VeroE6/TMPRSS2 cells
(XenoTech)). The multiplicity of infection (MOI) will depend on the virus stock titer. The incubation time
can vary if using a reporter virus.

The assay can be performed in 96-well plates (medium throughput screening) or 384-well plates (high throughput screening).

Scope

2 Evaluation of the in vitro antiviral activity of drugs against coronaviruses.

Safety and Environment

3 Samples contain BSL-3 level high risk agents (viral) with infectious aerosol transmission potential.

4 All materials should be decontaminated before exit from the facility.

5 All activities and procedures are performed in BSC class III in combination with full-body, air supplied positive pressure protective suits with PAPR.

6 Use Peroxigard to disinfect all surfaces and materials inside and outside the BSC.

7 Always use a secondary container inside the BSL-3 to transfer plates from the BSC to the incubator.

8 In BLS-2 and BSL-3, pour the approved disinfectant in a reservoir for tip disinfection and liquid waste disposal.

Procedure

9 BSL-2 steps (all steps to be performed inside the BSC):

9.1 The day before the assay, cells are grown in DMEM + 10% FBS & 1% antibiotic / antimycotic.

9.2 Using the EL406 washer / liquid dispenser, seed 3,000 cells / well in 19 µL of DMEM + 10% FBS (384-well plates) or 10,000 cells / well in 100 µL of DMEM + 10% FBS (96-well plates).

9.3 Incubate the plates at 37°C 5% CO2 for 24h.

9.4 On the same day, create your protocol for dispensing compounds using Tecan or I.dot.

9.5 On the following day, thaw the DMSO stocks of compounds at room temperature and check color and solubility.

9.6 Remove the plates from the incubator and use DMSO stocks to dispense the drugs into the plates using the Tecan D300 or the Cellink I-Dot. The titration range for the drugs will depend on each assay. If using 10mM DMSO stocks, the regular titration range includes 10 points of 3-fold dilutions, with concentrations from 36µM to 0.0018µM. If using 1mM DMSO stocks, the regular titration range includes 10 points of 3-fold dilutions, concentrations from 3.6µM to 0.00018µM. A minimum of two replicates per each compound should be included in each plate (two technical replicates per assay).

9.7 Normalize all wells to DMSO, with 0.5% as the maximum limit per well. If using VeroE6/TMPRSS2 cells, a P-GP should be added to avoid the efflux of compounds. The regular P-GP inhibitor used is Elacridar (GF120918 MedChem Express), at 2µM / well.

9.8 Untreated / uninfected wells and untreated / infected control wells are included in each plate (8 each in 96-well plate and 32 each in 384-well plate).

9.9 After dispensing the compounds, incubate for 2 h at 37°C and 5% CO2 before infecting the cells.

Note: For the dispensing process using I.dot, please refer to manual

For the dispensing process using Tecan, create your protocol with the decided dilutions and titration range for the compounds.

9.10 For 96-well plates, use the following layout (4 compounds can be evaluated per plate):

	36uM	12uM	4uM	1.33uM	0.44uM	0.148uM	0.049uM	0.016uM	0.005uM	0.0018uM		
	1	2	3	4	5	6	7	8	9	10	11	12
A	Compound 1 (replicate 1)										Infected wells	
B	Compound 1 (replicate 2)											
C	Compound 2 (replicate 1)											
D	Compound 2 (replicate 2)											
E	Compound 3 (replicate 1)										Uninfected wells	
F	Compound 3 (replicate 2)											
G	Compound 4 (replicate 1)											
H	Compound 4 (replicate 2)											

9.11 For 384-well plate, use the following layout (16 compounds can be evaluated per plate):

			Concentration (uM)										Concentration (uM)											
			36	12	4	1.33	0.44	0.148	0.049	0.016	0.005	0.0018	36	12	4	1.33	0.44	0.148	0.049	0.016	0.005	0.0018		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	Uninfected wells		Compound 1 (replicate 1)										Compound 9 (replicate 1)										Infected wells	
B			Compound 1 (replicate 2)										Compound 9 (replicate 2)											
C			Compound 2 (replicate 1)										Compound 10 (replicate 1)											
D			Compound 2 (replicate 2)										Compound 10 (replicate 2)											
E			Compound 3 (replicate 1)										Compound 11 (replicate 1)											
F			Compound 3 (replicate 2)										Compound 11 (replicate 2)											
G			Compound 4 (replicate 1)										Compound 12 (replicate 1)											
H			Compound 4 (replicate 2)										Compound 12 (replicate 2)											
I	Infected wells		Compound 5 (replicate 1)										Compound 13 (replicate 1)										Uninfected wells	
J			Compound 5 (replicate 2)										Compound 13 (replicate 2)											
K			Compound 6 (replicate 1)										Compound 14 (replicate 1)											
L			Compound 6 (replicate 2)										Compound 14 (replicate 2)											
M			Compound 7 (replicate 1)										Compound 15 (replicate 1)											
N			Compound 7 (replicate 2)										Compound 15 (replicate 2)											
O			Compound 8 (replicate 1)										Compound 16 (replicate 1)											
P			Compound 8 (replicate 2)										Compound 16 (replicate 2)											

10 **From the following step, all the procedure should be performed inside BSL3**

10.1 Prepare the virus suspension in DMEM+10% FBS, according to the concentration of the viral stock and the MOI selected for each virus.

Note: To calculate the MOI, divide the number of viral particles in your stock (PFU/ml) by the number of cells seeded per well. Consider in your formula the total number of wells to be infected.

Note: If the infection is going to be performed manually, calculate your total volume of viral suspension including 1-2 ml extra. If the infection is going to be performed using Integra Viaflo, you should include 10-11 ml extra, to consider the dead volume of Integra reservoirs.

10.2 Mix well the virus suspension before transferring it gently to the Integra reservoir.

10.3 Infect the corresponding wells by dispensing 6.5 µL of the virus suspension per well in 384-well plates or 10µL per well in 96-well plates.

10.4 After dispensing the virus, incubate the plates at 37°C and 5% CO2 (black plates for 48h and white plates for 72h).

10.5 After 48h of incubation, remove the black plates from the incubator and add Formal fix to inactivate all viral particles: 25 µL per well in 384-well plate, or 100µL per well in 96-well plate (using Integra or by multichannel pipette).

10.6 Incubate the formalfix-containing plate at 4°C during 24h.

10.7 After this time, remove the plates from the BSL3 contained in double ziploc bags properly sprayed with peroxigard.

10.8 Apply washing and immunostaining steps for fluorescence detection (SOP_18).

10.9 After 72h of incubation, remove the white plates from the incubator and add Cell Titer Glo to measure the luminescence: 25 µL per well in a 384-well plate or 100 µL per well in a 96-well plate (using Integra or by multichannel pipette).

10.10 Seal the plate with a breath-easy sealing membrane and incubate the plate at RT protected from light for 10-15 mins.

10.11 Read and record the luminescence using the Infinite 200 Pro (with an Integration time of 300ms).

Note: For nLuc SARS-CoV-2 or nLuc MERS reporter virus use MOI 0.01.

First read: after 24h of incubation remove the first plate from the incubator and add 25 µL of Nano Glo reagent to each well (using Integra or by multichannel pipette). Seal the plate with the breath-easy sealing membrane and read the luminescence using the Infinite 200 Pro (with an integration time of 300 ms).

Note: Buffer and substrate should be mixed immediately before the use for the virus detection.

Second read: after 48h remove the second plate from the incubator and add 25 µL of Cell Titer Glo reagent to each well (using Integra or by multichannel pipette). Seal the plate with the breath-easy sealing membrane and cover the bottom of the plate with black tape before reading the luminescence using the Infinite 200 Pro (with an Integration time of 300ms).

Protocol: Cytotoxicity (CC50)

11 In addition to the compound screening plates, each compound should be evaluated for cytotoxicity (white plates for 72h).
For this, follow the steps described in section above prior to addition of virus, with the difference that compound-containing plates are incubated during 72hr instead of 2hr. In addition, all control wells in the plate will correspond to untreated / uninfected wells.

Quality Control

12 Mixing steps should be performed correctly before seeding the cells and before infecting with the virus, to obtain homogenous infection between the wells.

13 Check the volume in each well after using the EL406 and the Integra.

14 Check the cell's growth after 24h incubation, before dispensing compounds.

15 Check the CPE after 24h, 48h or 72h under the microscope before adding the reagents.

Analysis

16 Use GraphPad prism to normalize the data. Plot the curves as inhibitory response versus concentration, and determine the IC50, EC50 and CC50 values using a non-linear regression (curve fit).

Definitions

17 BSC: Biosafety Cabinet
PAPR: Powered Air Purifying Respirator
CO2 : Carbon Dioxide
CMC: Carboxymethylcellulose
MEM: Minimum Essential Media
DMEM: Dulbecco's Modified Eagle's Medium
PBS: Phosphate Buffer Saline
FBS: Fetal Bovine Serum
BSL-2: Biosafety Laboratory – Level 2
BSL-3: Biosafety Laboratory – Level 3
Tips: aerosol-barrier pipette tips
mL: milliliters
µL: microliters
min: minutes