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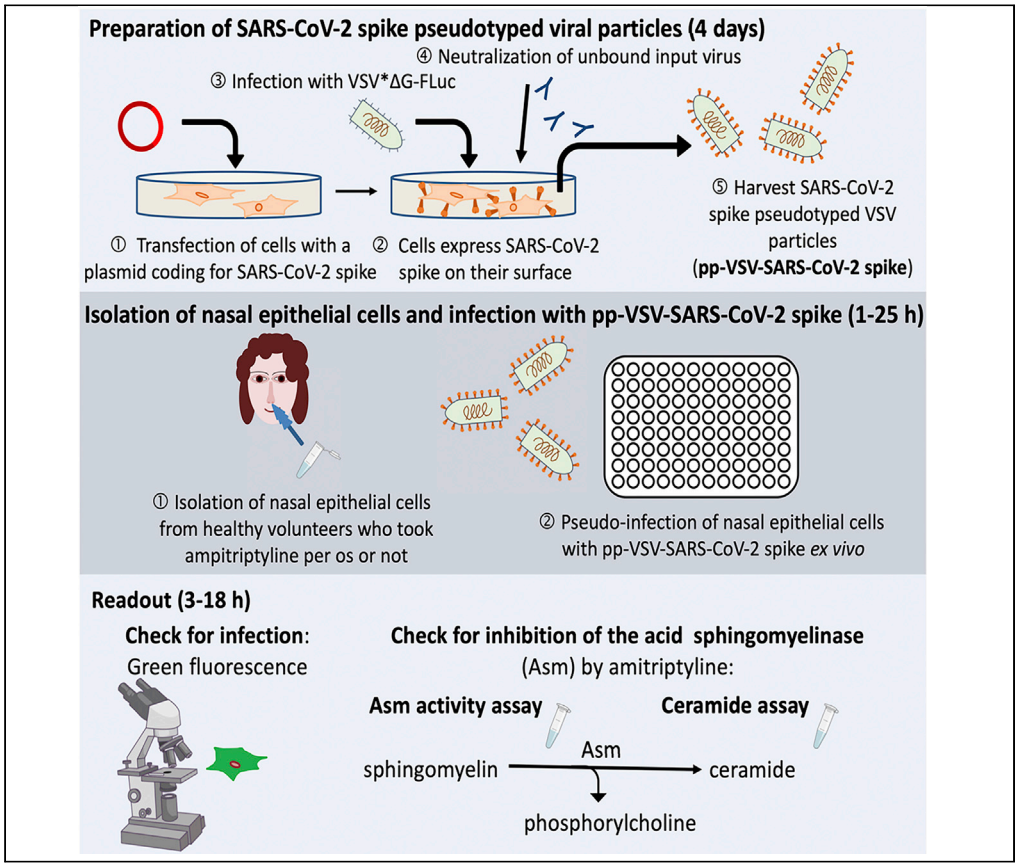
Ex vivo assay to evaluate the efficacy of drugs targeting sphingolipids in preventing SARS-CoV-2 infection of nasal epithelial cells



This protocol enables the testing of drugs against infection of epithelial cells with SARS-CoV-2 (severe acute respiratory syndrome coronavirus-2), using pseudo-typed replication deficient vesicular stomatitis virus particles (pp-VSV) presenting the SARS-CoV-2 spike protein. After treating human volunteers with amitriptyline, an approved antidepressant and inhibitor of the acid sphingomyelinase, freshly isolated nasal epithelial cells were infected *ex vivo* and infection levels were quantified. This protocol offers the possibility to rapidly test the efficacy of potential drugs in the fight against COVID-19.

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HIGHLIGHTS

SARS-CoV-2 spike pseudo-typed VSV particles allow to mimic SARS-CoV-2 infection

In vivo applied drugs can be tested *ex vivo* by infecting nasal epithelial cells

Infection can be easily determined by expression of eGFP

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Ex vivo assay to evaluate the efficacy of drugs targeting sphingolipids in preventing SARS-CoV-2 infection of nasal epithelial cells

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SUMMARY

This protocol enables the testing of drugs against infection of epithelial cells with SARS-CoV-2 (severe acute respiratory syndrome coronavirus-2), using pseudo-typed replication deficient vesicular stomatitis virus particles (pp-VSV) presenting the SARS-CoV-2 spike protein. After treating human volunteers with amitriptyline, an approved antidepressant and inhibitor of the acid sphingomyelinase, freshly isolated nasal epithelial cells were infected *ex vivo* and infection levels were quantified. This protocol offers the possibility to rapidly test the efficacy of potential drugs in the fight against COVID-19.

For complete details on the use and execution of this protocol, please refer to Carpinteiro et al. (2020).

BEFORE YOU BEGIN

The following protocols are described:

- Preparation and storage of pseudo-typed VSV particles presenting SARS-CoV-2 spike
- Treatment of healthy volunteers
- Isolation of nasal epithelial cells
- *Ex vivo* treatment of nasal epithelial cells
- Infection of freshly isolated nasal epithelial cells with SARS-CoV-2 spike pseudo-typed particles
- Acid sphingomyelinase (Asm) activity assay to check for inhibition of Asm by amitriptyline
- Quantification of ceramide

⚠ CRITICAL: When planning the experiments, it is mandatory to get an approval of the local ethics committee for treating human volunteers with experimental drugs and nasal epithelial cell isolation and a written informed consent of the volunteers.

Note: We used amitriptyline in our studies (Carpinteiro et al., 2020). Amitriptyline is approved for human use for depression by the European Medicines Agency (EMA) and Food and Drug Administration (FDA). Experience has been available for decades; amitriptyline is a well-tolerated drug if the dosage recommendations are followed and the contraindications are observed. The special legal requirements for the use of drugs in humans must be observed.

Note: For both assays, to determine Asm activity and quantify ceramide, radiolabeled biomolecules are used. Working with radioactivity requires special laboratories and a permission to use radionucleotides.

Note: Before you start with the experiments, make sure that all media and buffers are prepared. Media should be stored at 4°C and warmed up to 37°C before use. Buffers are stored at 4°C if not indicated otherwise.

Note: A few buffers are mixed directly before use as indicated below.

Freshly isolated human nasal epithelial cells are directly infected.

Note: All experiments with cells (cultured and freshly isolated primary cells) are performed under sterile conditions using a standard flow bench, and cells are incubated in a standard tissue culture incubator at 37°C with 5% CO_2.

Note: Samples to determine the acid sphingomyelinase activity and ceramide amount can be shock-frozen and stored at -80°C until the day when the assays are performed.

Note: For all experimental procedures, we use Millipore water if not indicated otherwise.

Note: The room temperature in our laboratories is 22°C. Even if the protocol below states 22°C instead of the phrase room temperature, these steps can be performed at 19°C–25°C as well.

Preparation of pseudo-typed VSV particles presenting SARS-CoV-2 spike

Timing: 10–24 days

Note: Working with SARS-CoV-2 pseudo-typed VSV particles requires laboratories equipped for biosafety level 2.

Note: Steps 1 to 3 are preparatory steps that are not necessarily to be done every time you prepare pseudotypes.

1. Preparation of VSV*ΔG-FLuc and VSV-G stocks [timing: 3 days]
   a. Start new BHK-21(G43) cell culture. These cells express VSV-G protein if induced by mifepristone.
   b. Add VSV*ΔG-FLuc stock to produce new VSV*ΔG-FLuc or add VSV-G stock to prepare new VSV-G stock.
   c. Collect VSV*ΔG-FLuc or VSV-G stock containing supernatant.
2. Titration of VSV*ΔG-FLuc or VSV-G stocks [timing: 3 days]
   a. Use HEK293T cells in 96-well plates.
   b. Prepare 10-fold serial dilutions of VSV*ΔG-FLuc and VSV-G stocks to be tested.
   c. Incubate cells with dilutions for 1 h.
   d. Determine titers by counting eGFP-positive cells.
3. Production of anti-VSV-G antibody [timing: 10–14 days]

a. Start new CRL-2700 cell culture (these mouse hybridoma cell line produces anti-VSV-G antibodies).
b. Reduce FBS concentration in the culture medium successively during cell culture period of about 10 days.
c. Collect antibody containing supernatant and use concentrator columns to concentrate.
d. Store antibody concentrate at -80°C.
e. Check for antibody concentration once per batch.

4. VSV pseudotype preparation [timing: 4 days]
   a. Use HEK293T cells.
   b. Transfect cells with plasmid-DNA coding for SARS-CoV-2 spike.
   c. Infect cells with VSV*ΔG-FLuc for 1 h.
   d. Collect pseudotype containing supernatant (pp-VSV-SARS-CoV-2 spike).
   e. Use pseudotypes directly or store at -80°C.

Treatment of healthy volunteers and nasal epithelial cell isolation

Timing: 25 h

5. First, isolate nasal epithelial cells from (untreated) healthy volunteers and prepare aliquots for all experimental assays you want to do.
6. Infect the freshly isolated cells with pp-VSV-SARS-CoV-2 spike or leave them untreated.
7. Healthy volunteers take amitriptyline orally.
8. 1.5 and 24 h after medication, isolate nasal epithelial cells again. Aliquot for the experiments you want to do.
9. Infect these epithelial cells with pp-VSV-SARS-CoV-2 spike.
10. 24 h after infection, check for eGFP expression of cells.

Acid sphingomyelinase (Asm) activity assay

Timing: 2–3 h

Note: This assay uses radionucleotides. Work requires special laboratories and the permission for handling radioactivity.

11. Lyse cell samples and dry the substrate for Asm assay, [^{14}C]sphingomyelin.
12. Resuspend dried substrate in Asm assay buffer and add diluted samples.
13. Incubate for 1 h at 37°C.
14. Stop reaction by adding organic solvents.
15. Separate phases and quantify the radioactive labeled, hydrophilic product of Asm reaction, [^{14}C]phosphorylcholine, in a scintillation counter.

Ceramide quantification

Timing: 6–12 h

Note: This assay uses radionucleotides. Work requires special laboratories and the permission for handling radioactivity.

16. Extract lipids of cell samples and thaw [^{32}P]γATP.
17. Prepare a mix containing lipids, kinase, [^{32}P]γATP and buffer.
18. Incubate for 1 h to phosphorylate cellular ceramide.
19. Extract lipids and separate samples by thin-layer chromatography (TLC).

20. Analyze the TLC plates with an appropriate imager and quantify ceramide in comparison to standard ceramide series.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-VSV-G antibody (I1, produced from CRL-2700 mouse hybridoma cells)	ATCC	Cat# CRL-2700; RRID: CVCL_G654
Bacterial and virus strains		
VSV*ΔG-FLuc	(Berger Rensch and Zimmer 2011)	N/A
Biological samples		
Human nasal epithelial cells	Healthy volunteers	N/A
Experimental models: cells and cell lines		
BHK-21(G43) cells	Laboratory of Georg Herrler, Institute of Virology, University of Veterinary Medicine Hannover, Hannover, Germany(Hanika et al., 2005)	N/A
HEK293T cells	DSMZ	Cat# ACC-635: RRID CVCL_0063
CRL-2700 cells	ATCC	Cat# CRL-2700; RRID:CVCL_G654
Chemicals, peptides, and recombinant proteins		
Dulbecco's modified Eagle's medium (DMEM), high glucose	PAN-Biotech	Cat# P04-03550
DMEM	Pan-Biotech	Cat# P04-01548S1
DMEM/F-12, GlutaMAX with supplement (DMEM/F-12)	Thermo Fisher Scientific	Cat# 31331093 (500 mL)
Minimal essential medium (MEM)	Gibco	Cat# 21090-022 (500mL)
MEM, GlutaMAX supplement	Thermo Fisher Scientific	Cat# 41090036
Fetal bovine serum (FBS)	Sigma-Aldrich	Cat# F7524
HyClone fetal calf serum (FCS)	Fisher Scientific	Cat# SV30160.03 (Lot: RZG35920)
Zeocin	Invivogen	Cat# ant-zn-05
Hygromycin	Invivogen	Cat# ant-hg-1
Penicillin/streptomycin	PAN-Biotech	Cat# P06-07100
Non-essential amino acid (NEAA) solution (10×)	PAN-Biotech	Cat# P08-32100
Sodium pyruvate	Thermo Fisher Scientific	Cat# P04-43100
Mifepristone	Sigma-Aldrich	Cat# M8046
Na_2HPO_4 (water-free)	Roth	Cat# P030.2
HEPES	Roth	Cat# HN77.5
NaCl	Roth	Cat# 3957.2
KCl	Roth	Cat# HN02.3
$CaCl_2$	Roth	Cat# HN04.2
$MgCl_2$	Sigma-Aldrich	Cat# M0250 – 1KG
$MgSO_4$	Sigma-Aldrich	Cat# M1880 – 500G
Sodium acetate	Sigma-Aldrich	Cat# S2889
HCl	Honeywell Fluka	Cat# 30721-2.5L
Acetic acid	Merck	Cat# 1.00063.2511
Imidazol	Sigma-Aldrich	Cat# I-2399
Dithiothreitol (DTT)	Sigma-Aldrich	Cat# D9163-1G
Diethylenetriaminepentaacetic acid (DTPA)	Sigma-Aldrich	Cat# D1133
Ethylenediaminetetraacetic acid (EDTA)	Serva	Cat# 11280
Ethylene glycol tetraacetic acid (EGTA)	Roth	Cat# 3054.1
Glucose	Roth	Cat# X997.2
Chloroform	Millipore	Cat# 1.02445.2500

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Methanol	J.T. Baker	Cat# 8404.2500
Acetone	Roth	Cat# 9372.5
NP40	Sigma-Aldrich	Cat#0 I3021 – 100mL
N octyl glucopyranoside (OGP)	Sigma-Aldrich	Cat# 08001-5G
Cardiolipin	Avanti Polar Lipids	Cat# 840012P
Amitriptyline (for *in vivo* use)	Neuraxpharm	PZN 3343120
Amitriptyline (for *in vitro* use)	Sigma-Aldrich	Cat# A8404
[^{14}C]sphingomyelin (cholin-methyl-^{14}C)	ARC (American Radiolabeled Chemicals)	Cat# ARC 0772
[^{32}P]γATP (6,000 Ci/mmol)	Hartmann Radiochemicals	Cat# SCP-501-150
DAG kinase	ENZO	Cat# BML-SE100
Scintillation fluid Rotiszint eco plus	Roth	Cat# 0016.3
Recombinant DNA		
Plasmid: pCG1-SARS-2-S	(Hoffmann et al., 2020)	N/A
Plasmid: pCAGGS-VSV-G	Brinkmann et al., 2017)	N/A
Software and algorithms		
ZEN imaging software	Zeiss	https://www.zeiss.de
Leica LCS (version 2.61)	Leica	https://www.leica.com
Quantasmart 3.04	PerkinElmer	https://www.perkinelmer.com
Image Reader FLA-3000 series V1.8	FUJIFILM	http://www.fujifilm.de
MultiGauge V3.0	FUJIFILM	http://www.fujifilm.de
Other		
Pipette tip 0.5–20 µL	Ratiolab	Cat# 2500170
Pipette tip 200 µL	Sarstedt	Cat# 70.760.002
Pipette tip 1,000 µL	Sarstedt	Cat# 70.3050
Serological Pipette 1 mL	Corning inc.	Cat# 4485
Serological Pipette 5 mL	Greiner Bio-One	Cat# 606180
Serological Pipette 10 mL	Greiner Bio-One	Cat# 607180
Serological Pipette 25 mL	Greiner Bio-One	Cat# 760180
1.5 mL microcentrifugation tube	Sarstedt	Cat# 72.690.001
2.0 mL microcentrifugation tube	Eppendorf	Cat# 0030120094
96-well plates	Sarstedt	Cat# 83.3924
T-75 cell culture flasks	Greiner Bio-One	Cat# 658175
T-175 cell culture flasks	Greiner Bio-One	Cat# 660175
Vivaspin Protein Concentrator Columns, 30 kDa MWCO	Sartorius	Cat# VS2021
Scintillation vials	Ratiolab	Cat# 5810100
Nose brush	Celletta brush	Cat# 8628001-01
Bottle top filter	TPP	Cat# 99505
Silica G60 thin-layer chromatography (TLC) plates	Supelco (Merck/Sigma-Aldrich)	Cat# 1.05748.0001
Glass cover slips (Ø 12 mm)	Menzel-Gläser/ ROTH	Cat# P231.1
Microscope slides (3 × 1 inch)	Engelbrecht	N/A
Simultaneous TLC chamber	Macherey-Nagel	Cat# 814019
Confocal microscope	Zeiss	LSM800 confocal laser scanning microscope
	Leica	TCS SL
	Leica	TCS SP5
Thermo shaker	Kisker Biotech or Eppendorf	TS100 Thermomix compact
Vacuum concentrator	Thermo Scientific	SpeedVac Concentrator Savant DNA120
Bath sonicator	Bandelin Electronic	Bandelin Sonorex RK100
Tri-Carb liquid scintillation analyzer	PerkinElmer	Tri-Carb2810TR
Phospho Imager	FUJIFILM	FLA-3000

MATERIALS AND EQUIPMENT

Medium for BHK-21(G43) cells		
Reagent	Final concentration	Amount
DMEM, High glucose	N/A	500 mL (1 bottle)
FBS	5%	25 mL
Zeocin (every 4th passage)	100 μg/mL	N/A
Hygromycin (every 4th passage)	50 μg/mL	N/A
TOTAL	N/A	535 mL

Note: For induction of VSV-G expression, add mifepristone at a final concentration of 10^{-8} M for 6 h.

Medium for HEK293T cells		
Reagent	Final concentration	Amount
DMEM	N/A	500 mL (1 bottle)
FBS	10%	50 mL
Penicillin	100 U/mL	N/A
Streptomycin	0.1 m/mL	N/A
TOTAL	N/A	560 mL

Medium for CRL-2700 cells		
Reagent	Final concentration	Amount
DMEM/F-12	N/A	250 mL (1/2 bottle)
MEM, GlutaMAX	N/A	250 mL (1/2 bottle)
FBS	10%	50 mL
Penicillin	100 U/mL	N/A
Streptomycin	0.1 m/mL	N/A
TOTAL	N/A	560 mL

Medium for human epithelial cells		
Reagent	Final concentration	Amount
MEM	N/A	500 mL (1 bottle)
FCS (Fisher Scientific)	10%	50 mL
TOTAL	N/A	550 mL

1× Phosphate buffered saline: 1× PBS buffer		
Reagent	Final concentration	Amount
NaCl	137 mM	8.01 g/L
KCl	2.7 mM	200 mg/L
KH_2PO_4	1.15 mM	156 mg/L
Na_2HPO_4 (water-free)	6.5 mM	1.157 g/L
TOTAL	N/A	1 L
Adjust pH to 7.4, sterilize by autoclaving and store at 22°C.		

2× HEPES buffered saline: 2× HBS buffer:		
Reagent	Final concentration	Amount
NaCl	280 mM	16.363 g/L
HEPES	50 mM	11.916 g/L
KCl	20 mM	1.491 g/L
Na_2HPO_4 (water-free)	1.5 mM	0.213 g/L
TOTAL	N/A	1 L
Adjust pH to 7.08–7.12, sterilize by filtration through 0.22 μm filter and store at 4°C.		

HEPES buffered saline: H/S buffer		
Reagent	Final concentration	Amount
NaCl	132 mM	26.4 mL 5 M NaCl
HEPES	20 mM	20 mL 1 M HEPES, pH 7.4
KCl	5 mM	5.4 mL 1 M KCl
$CaCl_2$	1 mM	1 mL 1 M $CaCl_2$
$MgCl_2$	0.7 mM	0.7 mL 1 M $MgCl_2$
$MgSO_4$	0.8 mM	0.8 mL 1 M $MgSO_4$
		Adjust pH to 7.3
H_2O		Add up to 1 L
TOTAL	N/A	1 L
Use a 0.22 μm bottle top filter unit to sterilize.		

Asm lysis buffer		
Reagent	Final concentration	Amount
Sodium acetate	250 mM	2.5 mL 1 M sodium acetate, pH 5
NP40	2%	2 mL 10% NP40
H_2O		5.5 mL
TOTAL	N/A	10 mL

Asm assay buffer		
Reagent	Final concentration	Amount
Sodium acetate	250 mM	2.5 mL 1 M sodium acetate, pH 5
NP40	0.1%	100 μL 10% NP40
H_2O		7.4 mL
TOTAL	N/A	10 mL

Asm dilution buffer		
Reagent	Final concentration	Amount
Sodium acetate	250 mM	2.5 mL 1 M sodium acetate, pH 5
H_2O		7.5 mL
TOTAL	N/A	10 mL

Lipid extraction mixture (Asm activity assay)		
Reagent	Final concentration	Amount
Chloroform	66%	2 of 3 vol. parts of end volume
Methanol	33%	1 of 3 vol. parts of end volume
TOTAL	N/A	e.g., 9 mL

Note: Mix directly before use!

Lipid extraction mixture (DAG kinase assay)		
Reagent	Final concentration	Amount
Chloroform	49.75%	5 mL chloroform
Methanol	49.75%	5 mL methanol
HCl	0.5%	50 µL HCl
TOTAL	N/A	10.05 mL

Note: Mix directly before use!

Detergent solution (DAG kinase assay)		
Reagent	Final concentration	Amount
OGP	7.5% (w/v)	250 mg OGP are added to 3.3 mL of the mixture of cardiolipin/DTPA (see directly below).
Cardiolipin DTPA	5 mM 1 mM	Dissolve 100 mg cardiolipin in 13.8 mL 1 mM DTPA, pH 6.6 by sonication and incubating at 37°C. 3.3 mL of this solution is used to mix it with OGP.
TOTAL	N/A	3.3 mL

Note: Buffer can be stored at -20°C.

Kinase buffer (DAG kinase assay)		
Reagent	Final concentration	Amount
Imidazol/HCl, pH 6.6	0.1 M	4 mL of 1 M Imidazol/pH 6.6
NaCl	100 mM	1 mL of 4 M NaCl
$MgCl_2$	25 mM	1 mL of 1 M $MgCl_2$
EGTA	2 mM	816 µL of 98 mM EGTA
H_2O		33.184 mL H_2O
TOTAL	N/A	40 mL

Note: Buffer can be stored at -20°C.

Kinase (DAG kinase assay)		
Reagent	Final concentration	Amount
DAG kinase	1 mg/10 mL	1 mg
Kinase buffer		10 mL
TOTAL	N/A	10 mL

Note: Aliquot the dissolved kinase solution and store at -20°C.

Complete salt solution (DAG kinase assay)		
Reagent	Final concentration	Amount
NaCl	135 mM	1.687 mL of 4 M NaCl
$CaCl_2$	1.5 mM	75 µL of 1 M $CaCl_2$
$MgCl_2$	0.5 mM	25 µL of 1 M $MgCl_2$
Glucose	5.6 mM	280 µL of 1 M glucose
HEPES, pH 7.2	10 mM	500 µL of 1 M HEPES, pH 7.2
H_2O		2.433 mL H_2O
TOTAL	N/A	5 mL

Note: Solution can be stored at -20°C.

TLC running buffer (DAG kinase assay)		
Reagent	Final concentration	Amount
Chloroform ($CHCl_3$)	50%	50 mL $CHCl_3$
Acetone	20%	20 mL acetone
Methanol (CH_3OH)	15%	15 mL CH_3OH
Acetic acid	10%	10 mL acetic acid
H_2O	5%	5 mL H_2O
TOTAL	N/A	100 mL

Note: Mix directly before use.

⚠ CRITICAL: Both assays for testing the efficacy of amitriptyline to inhibit the acid sphingomyelinase, the Asm activity assay, and the quantitative determination of ceramide require radiolabeled biomolecules. Working with radioactivity requires special laboratories and a permission to work with radionucleotides.

Alternatives:

a. The Asm assay can also be performed with fluorescence labeled sphingomyelin (e.g., BODIPY FL C_{12}-Sphingomyelin; Thermo Fisher Scientific; Cat# D7711).
b. Ceramide quantification can be performed by mass spectroscopy, alternatively.
c. Viral VSV-ΔG-FLuc and VSV-G stocks as well as the antibody against VSV-G are commercial available from Kerafast. When using this material, you can directly begin with step 26 of the detailed protocol below.

All these alternatives were not used in our studies and are not described within this protocol.

STEP-BY-STEP METHOD DETAILS

Preparation of pp-VSV-SARS-CoV-2 spike pseudo-typed viral particles

Timing: 14 days (preparatory steps) and 4 days (preparation of pseudotypes)

Pseudo-typed viral particles are based on a replication-restricted vesicular stomatitis virus (VSV) that codes for an enhanced green fluorescent protein (eGFP) and firefly luciferase (FLuc) instead of the parental VSV glycoprotein (VSV-G), VSV*ΔG-FLuc (Berger Rensch and Zimmer 2011). Due to the inability of VSV*ΔG-FLuc to express VSV-G, this virus is unable to produce infectious progeny virions in the absence of *in trans* expression of VSV-G (or another suitable viral surface glycoprotein). Therefore, pseudo-typed VSV particles that harbor viral surface glycoproteins allow for a single infectious cell entry event into target cells and are thus safe surrogate vectors for studying cell entry of highly pathogenic viruses without the need to work with the authentic virus. Pseudo-typing of VSV*ΔG-FLuc was performed according to a previously published protocol (Kleine-Weber et al., 2019).

Preparation of VSV*ΔG-FLuc (VSV-G) stocks using BHK-21(G43) cells

Timing: 3 days

1. Start new BHK-21(G43) cell culture. One day prior to infection, seed BHK-21(G43) cells into T-75 flask in 10 mL medium (10% FBS). Grow cells at 37°C with 5% CO_2 in a standard tissue incubator.

2. Once cells reach 75% confluence (typically achieved at 24 h post seeding), remove old culture medium and add new culture medium containing 10^{-8} M mifepristone and incubate for 6 h.
3. Remove medium and immediately add 8 mL DMEM containing diluted (1:1,000) VSV*ΔG-FLuc (or alternatively VSV-G is used as positive control) stock, incubate 1 h at 37°C.
4. Remove medium, wash 2–3 times gently with PBS.
5. Add 20 mL DMEM (high glucose) and 5% FCS supplemented with 10^{-8} M mifepristone and incubate for 16–24 h at 37°C with 5% CO_2 (until most of the cells are rounded and detached).
6. Collect supernatant, centrifuge to pellet cellular debris for 10 min at 2,000 × *g* at 22°C.
7. Prepare aliquots of 1 mL in cryo vials, flash-freeze samples and store at -80°C.

Titration of VSV*ΔG-FLuc (VSV-G) stocks using HEK293T cells

Timing: 3 days

8. One day prior to infection, seed HEK293T cells into 96-well plates (3×10^4 cells/well). Grow cells at 37°C with 5% CO_2 in a standard tissue incubator. Cells should reach 75% confluence (typically achieved at 24 h post seeding) for infection.
9. On the day of infection, prepare 10-fold serial dilutions of VSV*ΔG-FLuc (or alternatively VSV-G) stock by mixing of 900 μL culture medium with 100 μL of VSV*ΔG-FLuc (or alternatively VSV-G) stock (or previous dilution). Remove old culture medium and inoculate serially diluted VSV*ΔG-FLuc(VSV-G) onto cells (in technical triplicates or quadruplicates).
10. Incubate cells for 1 h at 37°C with 5% CO_2.
11. Remove medium, wash 2–3 times gently with PBS.
12. Add 100 μL of culture medium per well and incubate for 16–24 h at 37°C with 5% CO_2.
13. Count eGFP-positive cells using a fluorescence microscope. Ideally, wells containing 20–200 eGFP-positive cells should be counted. Counting is typically done by eye but can also be performed using an automated fluorescence reader (e.g., Bioreader-7000-F from Bio-Sys). Calculate titer of VSV*ΔG-FLuc (VSV-G) stocks according to the following formula:

Titer [ffu/mL] = average no. of eGFP-positive cells (of technical triplicates) × reciprocal serial dilution factor (dilution in which eGFP-positive cells were counted) × inoculum dilution factor (10, since 100 μL were used for inoculation)].

Production of anti-VSV-G antibody (I1) supernatants

Timing: 10–14 days

14. Start new CRL-2700 cell culture. Seed low (up to 10th) passage CRL-2700 cells at a density of 1×10^4 cells/mL into T-7[L] \1ask in 10 mL medium (10% FBS). Grow cells at 37°C with 5% CO_2 in a standard tissue incubator. Cell culture flasks should be kept in an upright position in the incubator in order to allow addition of cell culture medium volumes >20 mL (e.g., step 16).
15. When the cell count reaches 1×10^6 cells/mL, split cells 1:4 in order to obtain 4× T-75 flasks of CRL-2700 cells.
16. Continue cultivation until the density reaches 1×10^6 cells/mL. Add 30 mL of FBS-free culture medium (final FBS concentration = 2.5%).
17. Continue cultivation until the density reaches 1×10^6 cells/mL. Collect all cultures (= 160 mL). Add 240 mL of FBS-free culture medium (final FBS concentration = 1%) and seed 40 mL of cell suspension into 10 × T-175 flasks.
18. Continue cultivation until the density reaches 1×10^6 cells/mL. Collect all cultures (= 400 mL) and transfer into 50 mL sterile centrifugal tubes.
19. Centrifuge for 10 min at 600 × *g* to pellet cells (cells can be aliquoted in MEM/DMEM + 10% FBS + 10% DMSO and frozen), collect supernatant in a separate tube.

20. Load supernatant on Vivaspin Protein Concentrator Columns (molecular weight cut-off: 30 kDa) according to the manufacturer's instructions and centrifuge (4,000 × *g*/4°C) until the total volume has been reduced to ~40 mL (~ 10-fold concentrated).
21. Aliquot concentrated supernatant in cryo vials and store samples at -20°C (or -80°C).

Note: For determination of the appropriate dilution of the antibody supernatant to neutralize VSV-G, following procedure is done once per batch:

22. Seed HEK293T cells into 96-well plates (3 × 10^4 cells/well). Grow cells at 37°C with 5% CO_2 in a standard tissue incubator. Cells should reach 75% confluence for infection.
23. On the day of infection, pre-incubate VSV*ΔG-FLuc (VSV-G) (~10,000 ffu/50 μL) with 50 μL of 2-fold serial dilutions of the antibody preparation for 1 h at 37°C (technical triplicates or quadruplicates); Range: 1:100 to 1:6,400.
24. Remove culture supernatant from HEK293T cells, add 100 μL/well of VSV*ΔG-FLuc (VSV-G)/antibody mixtures and incubate for 16–24 h.
25. Check for eGFP-positive (infected) cells using a fluorescence microscope. The highest antibody dilution that leads to complete block of infection (no eGFP-positive cells) is the concentration that should be used for VSV pseudotype preparation.

VSV pseudotype preparation using HEK293T cells

Timing: 4 days

26. Start new HEK293T cell culture. Seed low passage HEK293T cells into 6-well plates (3 × 10^5 cells/well). Grow cells for 24 h at 37°C with 5% CO_2 in a standard tissue incubator.

Note: It is recommended not to use cells that have been split for more than 15 times. Using lower passages ensures a good cell quality and cell growth rates.

27. Prepare transfection mixtures in sterile (autoclaved) 1.5 mL reaction tubes as follows: Mix 6–8 μg of plasmid-DNA (pCG1-SARS-2-S for experimental design or alternatively pCAGGS-VSV-G as positive control) per well and sterile ultrapure water to a final volume of 112.5 μL, then add 12.5 μL of sterile-filtered $CaCl_2$ (stock conc. 2.5 M) and mix. Add 125 μL of sterile-filtered 2× HBS buffer and vortex, incubate for 15 min at 22°C.

⚠ CRITICAL: Adding the 2× HBS dropwise can help to ensure a clear transfection mixture (see Troubleshooting Problem 1).

28. Mix by pipetting gently (not vortexing) and add the transfection complexes dropwise to the cells, incubate overnight (16–18 h) at 37°C with 5% CO_2 in a standard tissue incubator.
29. Remove supernatant and inoculate cells with 1 mL VSV*ΔG-FLuc (or VSV-G as positive control), MOI (multiplicity of infection) = 3. Incubate for 1 h at 37°C and 5% CO_2 in a standard tissue incubator.
30. Remove supernatant carefully wash 1–2 times with PBS.
31. Add fresh medium containing anti-VSV-G antibodies (except for cells transfected with VSV-G; add fresh medium without anti-VSV-G antibodies instead) incubate overnight (16–18 h) at 37°C with 5% CO_2 in a standard tissue incubator.
32. Collect supernatant, remove cellular debris by centrifugation at 2,000 × *g* for 10 min and transfer clarified supernatants into fresh tubes.
33. Use supernatants either directly for transduction of target cells or store supernatants at -80°C.

Drug treatment of human volunteers, isolation, and infection of human nasal epithelial cells

Timing: 25 h

The acid sphingomyelinase (Asm)/ceramide system plays an important role in many bacterial and viral infections (Grassmé et al., 2003; Grassmé et al., 2005). Asm catalyzes the hydrolysis of sphingomyelin to phosphorylcholine and ceramide. Many antidepressants functionally inhibit the Asm activity by displacing the enzyme from lysosomal membranes and thereby inducing Asm degradation (Hurwitz et al., 1994). Previously, we have shown that amitriptyline, an approved antidepressant used in clinical routine and a functional inhibitor of the acid sphingomyelinase (FIASMA) (Albouz et al., 1981; Hurwitz et al., 1994; Kornhuber et al., 2008), prevents infection with pseudo-typed replication deficient vesicular stomatitis virus particles presenting the SARS-CoV-2 spike protein (pp-VSV-SARS-CoV-2 spike) (Hoffmann et al., 2020; Carpinteiro et. al., 2020).

Isolation of nasal epithelial cells from healthy human

Timing: 0.5 h

Human nasal epithelial cells are obtained from healthy, adult volunteers. We did not observe any differences in infection between cells isolated from women or men. Therefore, isolation from both genders is feasible.

The first epithelial cell isolation should be performed before the oral administration of the drug, amitriptyline in the present study.

34. Transfer 1 mL prewarmed H/S buffer to each micro tube, one tube per volunteer.
35. Keep a 96-well plate ready.
36. Epithelial cells are removed from the nasal mucosa employing a small nose brush. To this end, insert the brush 1.5 to 2 cm into the nose and gently rotate the brush to collect epithelial cells.
37. The cells are directly released from the brush and resuspended in the prewarmed H/S buffer in the micro tube by careful rotating the brush.
38. Pipette the cell suspension up and down once to mix the cells and then split the volume of each sample into identical aliquots (see Figure 1) and transfer into a new microcentrifugation tube. Prepare aliquots as later needed.
39. Cells are pelleted by centrifugation (22°C, 830 × *g*) for 10 min and the supernatant is discarded.
40. For cellular infection continue with step 43 directly. For amitriptyline treatment *ex vivo* continue with step 50. Cells of another aliquot can be directly shock-frozen and stored at -80°C for Asm activity assay or continue immediately with step 52/56. Alternatively, cells of this aliquot can be used to determine ceramide amounts. To this end, continue with step 70 directly or shock-freeze cells and store at -80°C.

Pause point: Samples to determine Asm activity and ceramide can be shock-frozen and stored at -80°C.

Drug treatment of the volunteers

41. Directly after nasal cell isolation, human volunteers take amitriptyline orally (0.5 mg/kg).

Note: Amitriptyline is approved for human use for depression by the European Medicines Agency (EMA) and Food and Drug Administration (FDA). Amitriptyline is a well-tolerated drug if the dosage recommendations are followed and the contraindications are observed. The special legal requirements for the use of drugs in humans must be observed.

42. 1.5 and 24 h after medication intake, isolation of nasal epithelial cells is repeated, identical to steps 35–40.

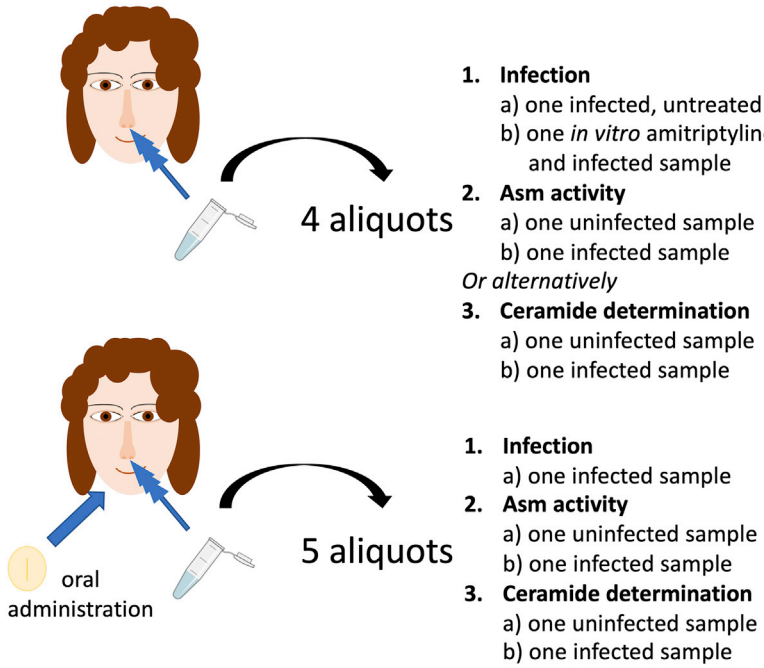

Figure 1. Overview of sample aliquoting and handling

Note: It is recommended to use the opposite nasal cavity for each successive epithelial cell collection to equalize the conditions.

Cellular infection with pp-VSV-SARS-CoV-2 spike and analyzation of infection efficacy

Timing: 0.5–25 h

43. Cells of two aliquots are resuspended in 100 µL prewarmed MEM/10% FCS, containing pp-VSV-SARS-CoV-2 spike (infected sample) and transferred into one well of a 96-well plate each.
44. Cells are incubated for30 (to determine Asm activity and ceramide amounts) or 60 min (to determine infection rates) at 37°C.
45. For determination of Asm activity after infection, cells are transferred into a micro tube, centrifuged, and washed once with H/S buffer as above. Discard supernatant and directly continue with section Acid sphingomyelinase activity (step 52/56) or shock-freeze the cells (without buffer) and store them at -80°C until the assay is performed. Alternatively, cells can be used to perform ceramide determination. In this case, continue with step 70 or freeze cells.

Pause point: Samples to determine Asm activity or ceramide can be shock-frozen and stored at -80°C.

46. Another aliquot of cells is also transferred into a micro tube, centrifuged, and washed once. Resuspend cells in 100 µL fresh MEM/10% FCS and culture them for 24 h to allow expression of the enhanced green fluorescence protein (eGFP) encoded by the particles.
47. Next day, transfer cells into a micro tube, spin down cells at 830 × *g* for 10 min, remove medium and resuspend cells in 20 µL H/S.
48. Pipet an aliquot of 10 µL onto a glass slide, cover with a cover slip and immediately visualize the cells on a fluorescence microscope.
49. Infection is determined by expression of eGFP. To this end, slides are analyzed with a confocal microscope (we are using Leica TCS SL or Leica TCS SP5) using a 40× lens and an adequate

software tool (e.g., Leica LCS software). At least 500 cells have to be counted for positive and negative eGFP expression in randomly chosen microscopic fields. Number of green fluorescent cells is set in relation to the total cells counted.

Ex vivo treatment of nasal epithelial cells with amitriptyline

Timing: 1.5 h

To compare efficacy of amitriptyline *in vitro*, one aliquot of freshly isolated nasal epithelial cells from untreated volunteers is treated with amitriptyline *ex vivo*.

Amitriptyline (Sigma-Aldrich) should be freshly prepared as 10 μM solution in 0.9% NaCl.

50. Cells of one aliquot are resuspended in 100 μL of a 10 μM amitriptyline solution and transferred into one well of a 96-well plate. Incubate for 60 min at 37°C in an incubator.
51. After incubation, cells are centrifuged as above, supernatant is discarded, and cells are resuspended in 100 μL prewarmed MEM/10% FCS, containing pp-VSV-SARS-CoV-2 spike. Continue with step 44 of section Cellular infection with pp-VSV-CoV-2 spike.

Check for the functional inhibition of the acid sphingomyelinase by amitriptyline

The antidepressant amitriptyline is a functional inhibitor of the enzyme acid sphingomyelinase (Asm). Asm catalyzes the degradation of sphingomyelin to phosphorylcholine and ceramide and plays a crucial role in many bacterial and viral infections, contributing to cellular entry of pathogens. We have previously shown that oral medication with amitriptyline by healthy volunteers inhibits the pseudo-infection of freshly isolated nasal epithelial cells with pseudo-typed VSV particles presenting SARS-CoV-2 spike on their surface (Carpinteiro et al., 2020).

Function of amitriptyline *in vivo* and *ex vivo* can be checked by using two different assays. The first assay determines the Asm activity by the use of an [^{14}C]labeled sphingomyelin, the substrate of the enzyme. The second assay determines one product of Asm reaction, intracellular ceramide amount, quantitatively.

Acid sphingomyelinase activity assay

Timing: 2–3 h

Note: Work on ice if not indicated otherwise.

Cell samples to determine Asm activity can be directly used or shock-frozen and stored at -80°C until the day of the assay. Centrifuge freshly prepared cell samples and discard the supernatant. Shock-frozen cell pellets should be quickly thawed at 37°C using a thermo shaker (see step 55).

As the substrate, radiolabeled [^{14}C]sphingomyelin is used. Assay readout is the release of hydrophilic [^{14}C]phosphorylcholine, separated by phase extraction from the lipophilic substrate.

Working with radionucleotides requires special laboratories and the permission for handling radioactivity.

52. Place 270 μL pre-cooled Asm dilution buffer into a new micro tube for each sample to be analyzed.
53. Remove [^{14}C]sphingomyelin from the freezer and pipette 0.1 μL [^{14}C]sphingomyelin, corresponding to 0.05 μCi, for each sample into one new micro tube.

Note: Always prepare sphingomyelin for the number of samples to be analyzed plus one (e.g., 20 samples means 2.1 μL ((20+1)*0.1 μL), corresponding to 1.05 μCi).

54. Dry the sphingomyelin resolved in organic solvent in a vacuum concentrator for 10 min (larger volumes may take longer).
55. Meanwhile, transfer frozen samples into a thermo shaker and thaw them quickly at 37°C.
56. Add 200 μL pre-cooled Asm lysis buffer to each sample and place samples on ice for 5 min.
57. In the meantime, continue with the dried substrate for the assay. Add 30 μL Asm assay buffer per original 0.1 μL to dried sphingomyelin (e.g., 20 samples means 630 μL assay buffer ([20+1] ×30 μL)) and sonicate in a sonication bath for 10 min to promote micelle formation.
58. Add 30 μL of lysed sample to pre-pipetted Asm dilution buffer to dilute NP40 concentration to 0.2% (final volume, V_{end} = 300 μL).
59. Add 30 μL of sonicated [^{14}C]sphingomyelin (in Asm assay buffer) to each sample (V_{end} = 330 μL).

Note: Sonicated sphingomyelin should not be vortexed or pipetted up and down before aliquoting.

60. Vortex each sample and transfer the tube into an 37°C prewarmed thermo shaker and incubate for 30 - 60 min at 300 rpm horizontal shaking and 37°C.
61. Enzymatic reaction is terminated by adding 800 μL of freshly mixed lipid extraction mixture ($CHCl_3$:CH_3OH, 2:1, v/v).
62. Vortex each sample for 30 s.
63. Separate hydrophilic and hydrophobic phases by centrifugation at maximum speed in a micro centrifuge for 5 min.
64. In the meantime pipette 4 mL scintillation fluid into a scintillation vial for each sample.
65. The radioactive labeled product is [^{14}C]phosphorylcholine that is in the upper hydrophilic phase and therefore can be separated from the lipophilic substrate [^{14}C]sphingomyelin which is in the lower lipid phase. Take an aliquot of the upper phase by pipetting twice 125 μL of the upper phase into prepared scintillation vials.

Note: This must be done very carefully without mixing the two phases or taking anything from the lower or intermediate phase.

66. Positive control: Add 20 μL of [^{14}C]sphingomyelin in Asm assay buffer (see step 2) to 4 mL scintillation liquid.Negative control: One scintillation vial with scintillation fluid, but without a sample.
67. Vortex each scintillation vial for 10 s and wait for 10 min before analyzing them in a scintillation counter.
68. Calculation of Asm activity:

Using the specific activity of [^{14}C]sphingomyelin, the counts of each sample are converted into the amount of product (moles). For calculation of the specific Asm activity (amount of product per time and amount of protein), the time of assay (30 - 60 min) and the amount of protein used has to be considered and specific enzyme activity is expressed as mol/mg/h. Protein determination can be performed with a commercial kit compared with a calibration protein series.

Optional: You can use the remaining lysed sample (in Asm lysis buffer; step 58; 170 μL) for ceramide determination. This approach saves sample, but requires a certain routine with methodology. Samples should be directly used or shock-frozen again.

Pause point: The rest of the samples can be shock-frozen and stored at -80°C and used for quantitative ceramide determination later (step 70).

Quantitative determination of ceramide

Timing: 6–12 h

Note: Cell samples to determine ceramide can be directly used or shock-frozen and stored at -80°C until the day of ceramide assay. Centrifuge freshly prepared cell samples and discard the supernatant. Shock-frozen cell pellets should be quickly thawed at 37°C using a thermo shaker.

To determine ceramide, [^{32}P]γATP is used to phosphorylate endogenous ceramide of the cell samples. Within the 60-min assay period the entire endogenous ceramide present in the samples is converted to radioactive ceramide-1-phosphate (and a fraction of non-radioactive ceramide). Readout is phosphorylated ceramide that can be quantified with an imager after thin-layer chromatography (TLC) and in comparison to standard ceramide series.

Since ^{32}P has a half-life of 28 days, current radioactivity has to be calculated and considered when pipetting the master kinase assay mix.

Working with radionucleotides requires special laboratories and the permission for handling radioactivity.

69. Prepare a TLC chamber with 100 mL of freshly prepared TLC running buffer, consisting of $CHCl_3$:C_3H_6O:CH_3OH:$C_2H_4O_2$:H_2O (50:20:15:10:5; v/v/v/v/v).
70. Resuspend each sample in 200 µL H_2O or use the rest of hydrophilic sample of the Asm activity assay (previous described method Acid sphingomyelinase activity, step 58).
71. Extract lipids by adding 600 µL lipid extraction mixture, consisting of $CHCl_3$:CH_3OH:1 N HCl (100:100:1; v/v/v), and vortex for 10 s.
72. For standards use 50, 100, 250, and 500 pmol of C16 and C24 ceramide each. Steps 70 and 71 are identical for the standard samples.
73. Separate phases by centrifugation for 5 min at 22°C and maximum speed.
74. Ceramide is in the lower, organic phase. Carefully transfer 180 µL (or more, if required) of the lower phase into a new 2 mL micro tube and dry the samples in a vacuum concentrator.
75. In the meanwhile, remove the [^{32}P]γATP from the freezer and let it thaw at 22°C (timing: ~ 1 h).
76. Prepare the kinase containing kinase assay master mix for the number of samples to be analyzed:

per sample:	50 µL kinase buffer
	10 µL DAG kinase
	10 µL 20 mM DTT solution
	0.7 µL 100 µM ATP (end concentration 1 µM)
	5 µCi [^{32}P]γATP

77. Re-dissolve dried samples (step 74) in 20 µL detergent solution.
78. Add 70 µL of master mix to each sample to start the kinase reaction.
79. Incubate for 60 min at 22°C with shaking on a horizontal mixer (350 rpm).
80. Stop kinase reaction by adding 1 mL lipid extraction mixture, containing $CHCl_3$:CH_3OH:1 N HCl (100:100:1; v/v/v), and vortex for 10 s.
81. Add 170 µL complete salt solution and vortex for 10 s.
82. Add 30 µL of 100 mM EDTA solution to promote phase separation and vortex once again.
83. Centrifuge the samples at 22°C for 5 min at maximum speed.
84. Transfer 2 times 170 µL of the lower phase, containing the phosphorylated ceramide, into a new 1.5 mL micro tube and dry the samples in a vacuum concentrator.

Note: Handle the samples carefully to avoid mixing phases and contaminating of your transfer probe with the upper, hydrophilic phase.

85. Re-dissolve dried samples in 20 μL freshly prepared $CHCl_3$:CH_3OH (1:1; v/v) and spot them in 3–4 μL steps onto a thin-layer chromatography (TLC) plate.

Note: During the spotting process, always close the sample tubes.

Note: Since the solvent evaporates quickly when spotted onto the TLC plate, it may be useful to place a sheet of squared paper at the bottom of the TLC plate for orientation of the sample application points. Choose a suitable, not too narrow distance between the spotting dots.

Note: Do not spot too many samples in parallel.

Note: The horizontal line of the application points should be 1 to 1.5 cm from the lower edge of the plate.

86. Use $CHCl_3$:C_3H_6O:CH_3OH:$C_2H_4O_2$:H_2O (50:20:15:10:5; v/v/v/v/v) as running buffer (step 1) to separate samples until the running front reached the end of the TLC plate.

Note: This takes about 3 h.

87. Analyze TLC plates with an appropriate imager and quantify ceramide spots in comparison to standard C16 and C24 ceramide series using an appropriate software tool.

EXPECTED OUTCOMES

Medications with potential benefit, inhibiting SARS-CoV-2 infections, should result in reduced cellular infections rates (less cells expressing eGFP) compared to untreated cells (more green fluorescent cells).

In contrast, only drugs inhibiting the acid sphingomyelinase should result in reduced Asm activity and reduced ceramide levels.

LIMITATIONS

The findings generated by these experimental systems do not necessarily reflect the clinical situation in COVID-19 patients. Clinical studies are required to define the use of the potential medications tested by other researchers. In fact, the potential use of antidepressants for the treatment of COVID-19 patients (Carpinteiro et al., 2020) was strongly supported by a recently pre-printed retrospective multicenter study in France demonstrating reduced risk of intubation and death of COVID-19 patients upon the use of antidepressants (Hoertel et al., 2020).

TROUBLESHOOTING

Problem 1

Calcium phosphate based transfection of HEK293T cells:

Cloudy transfection mixture (step 27).

Potential solution

Repeat the preparation of transfection mixture. It may be helpful to add the 2× HBS buffer dropwise while gently and constantly vortexing. The transfection mixture must be clear prior to its addition onto the cells. A clear solution contains small $Ca_3(PO_4)_2$ precipitates which is a prerequisite for

effective transfection. A cloudy transfection mixture indicates large $CaPO_4$ precipitates and will result in low transfection efficacy.

Problem 2

Nasal epithelial cell isolation:

Insufficient numbers of cells to analyze (steps 36, 37).

Potential solution

Either you have isolated insufficient numbers of cells (step 36) or the isolated nasal epithelial cells are not well released from the brush (step 37). Repeat the isolation procedure and make sure that the brush is inserted up to 2 cm. Sometimes it may helpful to press a finger onto that region of the nose where you want to place the brush. Also make sure that all cells are released from the brush by carefully rotating the brush at the inner side of the conical tube.

Problem 3

Asm activity assay:

Scintillation counts are not detectable (step 67).

Potential solution

The only possible explanation, besides a defect of your scintillation counter, is omitting to add the radiolabeled sphingomyelin. Repeat the complete assay.

Problem 4

Asm activity assay:

Scintillation counts of identically treated samples show large deviations (step 67).

Potential solution

This can happen when the samples were wiped with a dry paper towel before scintillation measurement leading to electrical charging. Let the samples stand for a while and repeat measurement.

Another explanation is the contamination of samples (upper, hydrophilic phase) with varying amounts of the lipophilic phase. When pipetting the upper phase, be very careful not to mix the phases. If you have any doubts concerning contamination or mixing of the phases, centrifuge the sample tubes once again. Start over again with step 63.

Problem 5

Quantitative ceramide determinations:

Either no or weak signals are detected on the TLC plates (step 87).

Potential solution

Calculation of aged [^{32}P]γATP is wrong. Calculate the amount of 5 μCi [^{32}P]γATP once again and repeat the complete assay with shock-frozen samples.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Katrin Anne Becker (katrin.anne.becker@uni-due.de).

Materials availability

Request for material and reagents to generate the rhabdoviral vectors used in this study can be directed to Markus Hoffmann (mhoffmann@dpz.eu) and Stefan Pöhlmann (spoehlmann@dpz.eu). It will be made available upon installment of a material transfer agreement (MTA). Please note that several reagents are available in similar designs from other manufacturers. In this manuscript, we describe the reagents that we used. Alternative reagents may work in the same way.

Data and code availability

No new datasets or codes were generated in the preparation of this manuscript.

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AUTHOR CONTRIBUTIONS

Conceptualization, K.A.B. and A.C.; Writing – Original Draft, K.A.B., A.C., and M.H.; Writing – Review & Editing, K.A.B., A.C., and M.H.; Funding Acquisition, E.G. and S.P.; Supervision, E.G., J.K., and S.P.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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