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Protocol for Screening Host-Targeting Antivirals (HTAs) Using Human PBMCs and pDCs

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Abstract

This protocol offers an ex vivo method for screening host-targeting antivirals (HTAs) using human peripheral blood mononuclear cells (PBMCs) or plasmacytoid dendritic cells (pDCs). Unlike virus-targeting antivirals (VTAs), HTAs provide advantages in overcoming drug resistance and offering broad-spectrum protection, especially against rapidly mutating or newly emerging viruses. By focusing on PBMCs or pDCs, known for their high production of humoral factors such as Type I interferons (IFNs), the protocol enables the screening of antivirals that modulate immune responses against viruses. Targeting host pathways, especially innate immunity, allows for species-independent antiviral activity, reducing the likelihood of viral escape mutations. Additionally, the protocol's versatility makes it a powerful tool for testing potential antivirals against various viral pathogens, including emerging viruses, positioning it as an essential resource in both pandemic preparedness and broad-spectrum antiviral research. This approach differentiates itself from existing protocols by focusing on host immune modulation through pDCs, offering a novel avenue for HTA discovery.

Key features

- Optimized protocol for screening HTAs against dengue virus (DENV), chikungunya virus (CHIKV), and Zika virus (ZIKV).
- This protocol is ideal for screening soluble or intravenous-formulated compounds for evaluating their efficacy in experimental settings.
- This protocol builds upon the method developed by Tsuji et al. [1] and extends its application to PBMCs

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and testing against DENV, CHIKV, and ZIKV.

Keywords: Host-targeting antivirals (HTA), Human peripheral blood mononuclear cells (PBMCs), Plaque assay, Foci-forming unit assay (FFUA), Immunophenotyping

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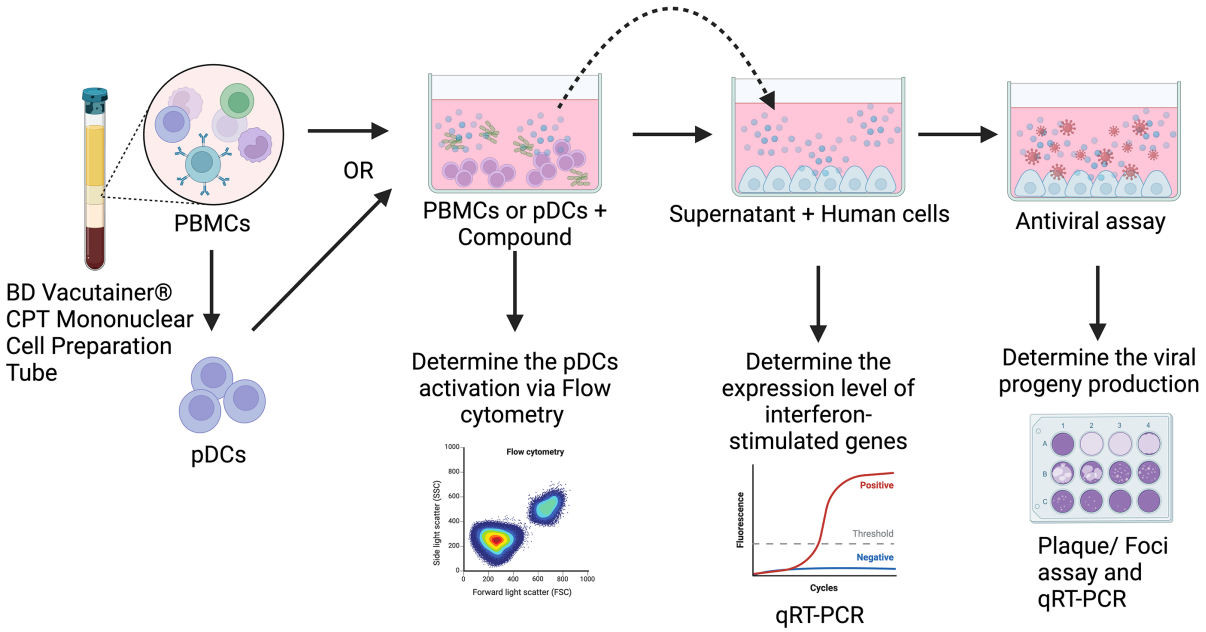
Graphical overview



Background

The prevalence of arboviruses like dengue (DENV), chikungunya (CHIKV), and Zika (ZIKV) underscores the need for innovative antiviral strategies [2–4]. Traditionally, virus-targeting antivirals (VTAs) have focused on viral components [5–8], which often lack broad-spectrum efficacy and are prone to resistance from rapidly mutating viruses due to their specificity. Host-targeting antivirals (HTAs), which target host proteins or pathways essential for viral replication, offer a promising alternative [9], providing a higher genetic barrier to resistance and broad-spectrum activity. The FDA-approved Maraviroc medication for HIV-1 highlights the growing importance of HTAs in antiviral research [10]. This manuscript presents an ex vivo model to screen HTAs using immune cells like peripheral blood mononuclear cells (PBMCs) or plasmacytoid dendritic cells (pDCs). It builds upon the method developed by Tsuji et al. [1] where bone marrow–derived dendritic cells (bmDCs) were stimulated with HTAs. Here, we use PBMCs, directly removing the need for advanced technical expertise and experimental animals associated with isolating bmDCs. PBMCs, which include lymphocytes, monocytes, and dendritic cells, produce a wide range of immune factors, making them physiologically relevant for studying immune-modulating antivirals [11]. The protocol uses BD Vacutainer CPT mononuclear cell preparation tubes for quick and efficient PBMC isolation within 1 h while preserving cell integrity [12]. Since

interferon (IFN) responses are critical to the first line of antiviral immunity, the protocol focuses on HTAs that activate isolated pDCs or pDCs within the PBMCs, which produce Type I interferons [13,14]. This method describes the isolation and culture of PBMCs or pDCs for HTA screening, involving the transfer of conditioned media from these immune cells to human liver cells (Huh-7) to assess the reduction in viral infectivity. It closely mimics physiological conditions, as HTAs in the bloodstream are likely to interact with immune cells like PBMCs or pDCs, producing humoral factors that travel to distant organs, and prime human cells for enhanced antiviral defense. Additionally, it introduces an immunophenotyping assay to assess the activation of pDCs and an IFN-stimulating genes (ISGs) expression study to evaluate the antiviral status of Huh-7 cells. These mechanistic assays are highly adaptable; while the current setup focuses on pDCs activation and ISGs induction, it can be modified to investigate other mechanisms by changing the primers used for gene expression analysis or selecting alternative immune cell surface markers to investigate other cells in the PBMCs such as monocytes and $CD8^+$ T cells. This flexibility ensures that the protocol can be tailored to specific research needs, making it a versatile tool in the study of HTAs. While the protocol provides a strong platform for screening HTAs, it lacks the complexity of in vivo models, limiting the ability to study interactions between different immune cells in a systemic context [15]. The conditioned media approach offers an indirect assessment of antiviral activity, missing some cellular interactions and immune responses seen in real infections. However, further in vivo evaluations using C57BL/6, AG129, and BALB/c are required to confirm the efficacy of the antiviral against DENV, CHIKV, and ZIKV before proceeding to clinical studies. Moreover, since immune cells must directly engage with the HTAs, tested drugs ideally need to be injectable to reach immune cells in the bloodstream. This limits the screening of oral or topical HTAs. However, repurposing injectable drugs with established safety profiles could expedite clinical use [16]. Future work should aim to address these limitations by incorporating in vivo models to provide a more comprehensive understanding of selected HTAs, investigating other immune pathways, and considering more diverse viral models and HTA candidates.

Materials and reagents

Biological materials

1. Human peripheral blood mononuclear cells (PBMCs) either isolated from healthy individuals or purchased from IQ Biosciences (catalog number: IQB-PBMC103) or ImmunoSpot by CTL (for specific PBMC selection, please visit https://immunospot.com/epbmc).
2. Vero African green monkey kidney cells (ECACC strain, ATCC CCL-81)
3. Huh-7 human hepatocytes (JCRB Cell Bank, JCRB0403)
4. DENV-2 (NGC, ATCC VR-1584)
5. CHIKV (ECSA, FN295485)
6. ZIKV (P6740, obtained from the University of Texas Medical Branch)

Reagents

1. Phosphate-buffered saline (PBS) (CANVAX, catalog number: BR0003)
2. Cellbanker2 (AMSBIO, catalog number: 11914)
3. Roswell Park Memorial Institute (RPMI) 1640 [+] l-glutamine (Corning, catalog number: MT-10-040-CV)
4. Sodium pyruvate (1 mM) (Gibco, catalog number: 11360-070)

5. 0.25% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (2.5 mM) (Gibco, catalog number: 15630-080)
6. Antibiotic-antimycotic (Gibco, catalog number: 15240-062)
7. Fetal bovine serum (FBS) (Gibco, catalog number: 10270-106)
8. Non-essential amino acid (NEAA) (Gibco, catalog number: 11140-050)
9. Dulbecco's modified Eagle's medium (DMEM) [+] 4.5 g/L glucose, l-glutamine, sodium pyruvate (Corning, catalog number: MT-10-013-CV)
10. Dulbecco's modified Eagle's medium (DMEM) [+] 1.5 g/L glucose, l-glutamine, sodium pyruvate (Corning, catalog number: MT-10-009-CV)
11. DMEM, powder, high glucose (Gibco, catalog number: 12100046)
12. Trypsin powder (Gibco, catalog number: 27250018)
13. Oligodeoxynucleotides (ODNs) containing unmethylated cytosine-guanine dinucleotides (CpG) (CpG ODN 2216) (InvivoGen, catalog number: tlrl-2216)
14. Pharmingen stain BSA buffer (staining buffer) (BD, catalog number: MAB554657)
15. CD304 HU NEUROPILIN-1 BB515 U21-1283 (BD, catalog number: PMG566036)
16. HU IL-3RALP (CD123) APC 6H6 (BD, catalog number: PMG567275)
17. HU CD86 PE IT2.2 (BD, catalog number: PMG555665)
18. ANTI-HLA-DR PE-CY7 L243 (G46-6) RUO/GMP (BD, catalog number: MAB335795)
19. 7-AAD viability dye, VIA-probe solution (BD, catalog number: MAB555816)
20. High viscosity carboxymethyl cellulose (CMC) (Sigma-Aldrich, catalog number: 9004-32-4)
21. Paraformaldehyde (MP Biomedicals, catalog number: 02150146-CF)
22. Triton X-100 (from any qualified supplier)
23. Skim milk (Chemiz, catalog number: 12947)
24. Anti-human HRP-conjugated secondary antibody IgG (H+L) (Invitrogen, catalog number: A18903)
25. TrueBlue peroxidase substrate (Seracare, catalog number: 5510-0030)
26. Crystal violet stain (from any qualified supplier)
27. RNeasy Kit (Qiagen, catalog number: QIAG-74106)
28. iScript cDNA Synthesis kit (Bio-Rad, catalog number: 1708891)
29. Luna universal qPCR master mix (New England BioLabs, catalog number: M3003L)
30. EasySep Human Plasmacytoid DC Isolation kit (STEMCELL Technologies, catalog number: 17977)
31. EasySep buffer (STEMCELL Technologies, catalog number: 20144)

Solutions

1. PBMC culture media (see Recipes)
2. Virus immobilizing media (see Recipes)
3. Cell culture growth media (see Recipes)
4. Cell culture maintenance media (see Recipes)

Recipes

1. PBMC culture media (50 mL)

Reagent	Final concentration	Quantity or Volume
Antibiotic-antimycotic (100×)	1×	500 µL

Sodium pyruvate (100 mM)	1 mM	500 µL
HEPES (1 M)	2.5 mM	125 µL
FBS (100%)	10%	5 mL
NEAA (100×)	1×	500 µL
RPMI 1640 medium (1×)	1×	43.375 mL (top up RPMI media to 50 mL)
Total		50 mL

2. Virus immobilizing media (500 mL)

Note: Dissolve 4.5 g of CMC in 250 mL of Milli-Q water. Autoclave the solution to sterilize and fully dissolve the CMC. Prepare 500 mL of 2× DMEM by dissolving one pack of DMEM powder in 500 mL of Milli-Q water, then filter to sterilize the media.

Reagent	Final concentration	Quantity or Volume
CMC (1.8%)	0.9%	250 mL
DMEM [+] 4.5 g/L glucose (2×)	1×	240 mL
FBS (100%)	2%	10 mL
Total		500 mL

3. Cell culture growth media (50 mL)

Reagent	Final concentration	Quantity or Volume
DMEM [+] 4.5 g/L glucose, l-glutamine, sodium pyruvate (for Vero cells growth culture) (1×)		
Or	1×	45 mL
DMEM [+] 1.5 g/L glucose, l-glutamine, sodium pyruvate (for Huh-7 cells growth culture) (1×)		
FBS (100%)	10%	5 mL
Total		50 mL

4. Cell culture maintenance media (50 mL)

Reagent	Final concentration	Quantity or Volume
DMEM [+] 4.5 g/L glucose, l-glutamine, sodium pyruvate (for Vero cells maintenance culture) (1×)	1×	49 mL
FBS (100%)	2%	1 mL
Total		50 mL

Laboratory supplies

1. BD Vacutainer CPT mononuclear cell preparation tubes (8 mL per tube) (BD, catalog number: SS362761)
2. 20-gauge (0.9 mm) needle (from any qualified supplier)
3. 96-well plate (from any qualified supplier)
4. 24-well plate (from any qualified supplier)
5. 2 mL internal threaded polypropylene cryogenic vial, self-standing with round bottom (Corning, catalog number: 431386)
6. 15 and 50 mL centrifuge tubes (from any qualified supplier)
7. 1.5 mL tubes (Eppendorf, catalog number: 022363204)

8. Falcon 5 mL round-bottom polystyrene test tube, with cell strainer snap cap (Corning, catalog number: 352235)

Equipment

1. Inverted microscope (Olympus, model: CKX31)
2. TC20 automated cell counter (Bio-Rad, catalog number: 1450102)
3. Refrigerated centrifuge (Eppendorf, catalog number: 5804 000.017)
4. Class II biological safety cabinet (BSC) (ESCO, model: SC2-4E1)
5. Flow cytometer with blue and red laser (BD, model: FACSCanto II)
6. NanoDrop 2000/2000c spectrophotometer (Thermo Fisher Scientific, catalog number: ND2000CLAPTOP)
7. Applied Biosystems™ SimpliAmp™ thermal cycler (Thermo Fisher Scientific, catalog number: A24811)
8. QuantStudio 5 real-time PCR system (Thermo Fisher Scientific, catalog number: A34322)
9. EasySep Violet Magnet (STEMCELL Technologies, catalog number: 18000)

Software and datasets

1. FACSDiva software (version 6.1.2, 23/09/2008)
2. Design & Analysis 1 (DA1) software (version 1.5.2)
3. EZR software program (version 1.63, 30/6/2024)

Procedure

A. PBMCs isolation, preservation, and culture

Note: PBMCs can either be isolated from healthy individuals with approval from an ethics committee or purchased as cryopreserved PBMCs from reputable sources, such as IQ Biosciences or ImmunoSpot by CTL. Below are the procedures for isolating PBMCs from healthy individuals.

1. Collect 16 mL of blood from each participant using two BD Vacutainer CPT mononuclear cell preparation tubes (8 mL per tube) containing buffered sodium citrate anticoagulant, liquid density medium, and an inert gel barrier (BD, USA).

Note: The number of CPT tubes used for PBMC isolation varies based on the required PBMC count for each experiment and the blood volume approved by the ethics committee. As a reference, using two CPT tubes yields 2.14 × 10^7 cells/mL ± 5.73 × 10^6 cells/mL with 91.16% ± 4.14% viability (N = 25), as measured by Trypan Blue staining using the TC20 Automated Cell Counter. If cell viability is low, consider using Benzonase, a DNase that effectively degrades extracellular DNA released from dead cells, thereby reducing the viscosity of the cell pellet and improving sample handling.

2. Centrifuge the tubes at 1,600× *g* for 30 min at room temperature within 2 h of blood collection.
3. After centrifugation, carefully pipette out the PBMCs, the whitish or cloudy layer (Figure 1), from both tubes without disturbing the gel barrier or other layers. Pool the PBMCs from the two tubes into a single sterile 50 mL tube.

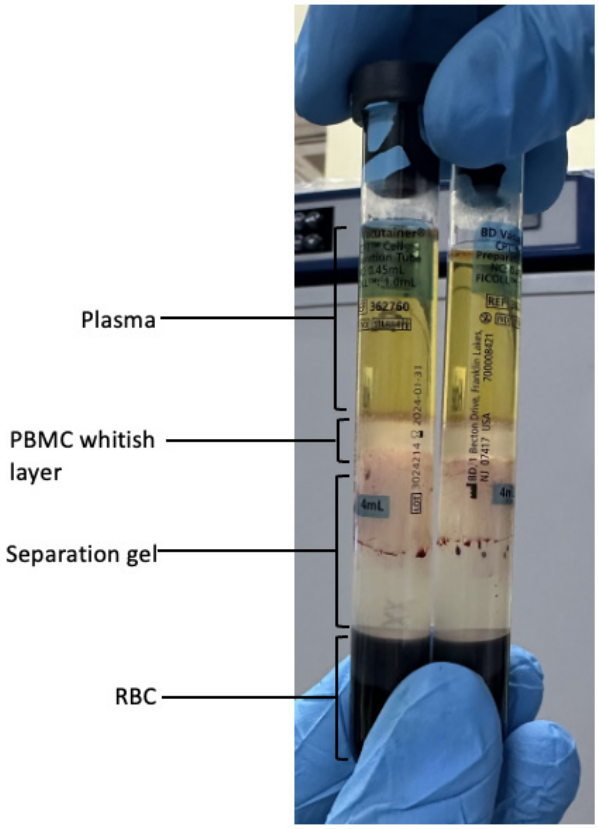


Figure 1. Peripheral blood mononuclear cell (PBMC) separation from plasma and red blood cells (RBC) using BD Vacutainer CPT mononuclear cell preparation tubes

4. Wash the pooled PBMCs twice. For each wash, resuspend the PBMCs in 30 mL of PBS and centrifuge at 300× *g* for 10 min at room temperature.
5. Discard the supernatant after each wash by careful decantation or aspiration using a serological pipette.
6. After the final wash, resuspend the PBMC pellet in 3 mL of Cellbanker2 for cryopreservation and aliquot the PBMC suspension into three cryovials, ensuring each vial contains 1 mL of cells.
Note: The volume of Cellbanker2 used to resuspend the PBMC pellet and the number of aliquots to be cryopreserved depends on the required PBMC count for each experiment. Generally, 1 mL of cell suspension is aliquoted into one cryovial.
7. Immediately store the PBMC vials at -80 °C for cryopreservation.
Note: Cryopreservation of PBMCs in Cellbanker2 enables long-term storage at -80 °C for over 2 years, eliminating the need for liquid nitrogen. However, if 10% DMSO is used as the cryoprotectant, storage in liquid nitrogen is required.
8. To thaw the PBMCs, place the cryovials in a 37 °C water bath until the cells are 80% thawed, ensuring the water does not reach the cap of the vial.
9. Transfer the thawed PBMCs into a sterile tube containing 10 mL of prewarmed PBMC culture media (see Recipe 1).
10. Centrifuge the cells at 300× *g* for 10 min to remove the cryoprotectant.
11. Carefully discard the supernatant.
12. Resuspend the PBMC pellet in PBMC culture media (see Recipe 1) and proceed to section E to stimulate and cultivate the immune cells.

B. pDCs isolation and purity evaluation

Note: Use EasySep Human Plasmacytoid DC Isolation kit to isolate pDCs from PBMCs [17]. The protocol from this section was used in a previous study by Ishii et al. [17].

1. Thaw the isolated PBMCs in a 37 °C water bath.
2. Transfer the thawed cells into 10 mL of PBS in a sterile 15 mL tube.
3. Gently mix by inverting the tube 5–10 times.
4. Centrifuge the cells at 300× *g* for 3 min at room temperature.
5. Discard the supernatant and resuspend the cell pellet in 1 mL of EasySep buffer.
6. Transfer the entire cell suspension into a 5 mL FACS tube.
7. Add 30 µL of anti-human CD32 (Fc gamma RII) Blocker, 50 µL of EasySep human plasmacytoid DC isolation cocktail component A, and 50 µL of EasySep human plasmacytoid DC isolation cocktail component B.
8. Mix well by gently pipetting.
9. Incubate at room temperature for 10 min.
10. During the incubation, vortex the EasySep Dextran RapidSpheres for 30 s.
11. After the incubation, add 50 µL of the EasySep Dextran RapidSpheres to the cell suspension.
12. Mix well by gently pipetting.
13. Incubate at room temperature for 1 min.
14. Add 1,320 µL of EasySep buffer to reach a final volume of 2.5 mL.
15. Mix well by gently pipetting.
16. Insert the FACS tube (without the lid) containing the cell suspension into the EasySep Violet Magnet.
17. Incubate at room temperature for 3 min.
18. Pick up the magnet and, in one continuous motion, invert the magnet holding the tube and pour the isolated pDCs suspension into a new tube.
19. Remove the tube from the magnet, place the new tube (without the lid) into the magnet, and incubate at room temperature for 3 min (second separation).
20. Pick up the magnet and, in one continuous motion, invert the magnet holding the tube and pour the isolated pDCs suspension into a new tube.
21. Isolated pDCs are ready to use for HTA screening. Proceed to section E to stimulate and cultivate the immune cells.

22. It is recommended to check the purity the first time of pDC isolation, following the instructions below. The result is presented in Figure 2.

a. After pDC isolation, wash the pDCs with 3 mL of staining buffer and then pellet them by centrifuging at 300× *g* for 3 min.
b. Resuspend the cell pellet in 200 µL of staining buffer.
c. Add 5 µL of CD304/BB515 and 5 µL of CD123/APC into each sample.
d. Incubate for 30 min on ice and away from a light source.
e. To remove unbound antibodies, wash the cells by adding 2 mL of staining buffer, gently mix, and centrifuge.
f. Resuspend the cells in 300 µL of staining buffer, add 20 µL of 7-AAD viability dye, and incubate on ice for 5 min.
g. Finally, analyze the cells using the BD FACSCanto II flow cytometer, and analyze data using FACSDiva software (BD, USA) (Figure 2).

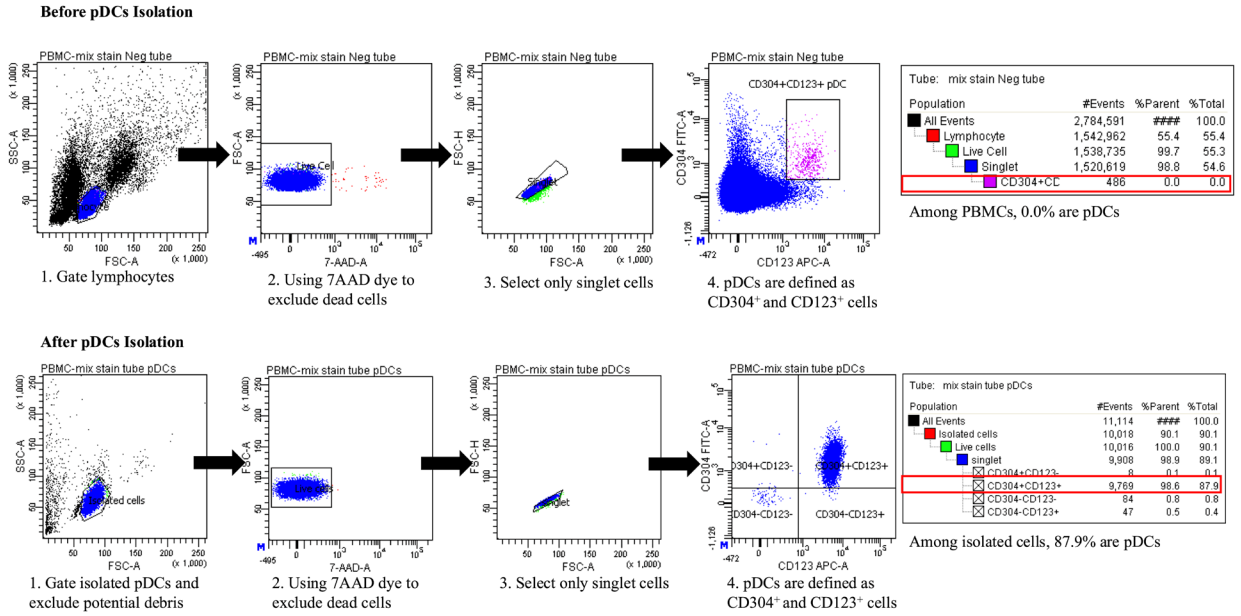


Figure 2. Immunophenotyping assay to access the purity of isolated plasmacytoid dendritic cells (pDCs). pDCs are defined as CD304$^+$/CD123$^+$ cells. The EasySep Human Plasmacytoid DC Isolation kit increased the pDCs percentage from 0% to 87.9%, highlighted in red boxes.

C. Cells

1. Culture Vero African green monkey kidney cells in high-glucose DMEM containing 4 g/L glucose supplement with 10% heat-inactivated FBS (see Recipe 3).
2. Culture Huh-7 human hepatocyte cells in low-glucose DMEM containing 1 g/L glucose, supplemented with 10% heat-inactivated FBS (see Recipe 3).
3. Maintain both cell lines in a 37 °C incubator with 5% CO_2 in a humidified atmosphere.
4. Monitor the cell cultures daily under a microscope for confluency and health.
5. Once the cells reach approximately 80%–90% confluency (typically every 3–4 days), subculture the cells.
6. For subculturing, aspirate the exhausted media and gently wash the cells with 10 mL of PBS that has been warmed to room temperature.
7. Add 2 mL of 0.25% trypsin-EDTA to the flask and incubate for 5 min at 37 °C to detach the cells.
8. Once cells are detached, add 5 mL of fresh DMEM (supplemented with 10% FBS) to neutralize the trypsin.
9. Transfer the cell suspension to a 15 mL conical tube and centrifuge at 300 × *g* for 3 min at room temperature.
10. Discard the supernatant and resuspend the cell pellet in 1 mL of fresh supplemented DMEM.
11. Re-seed the cells into new T75 flasks at the appropriate density for continued culture.

Notes:

1. For Vero cells, 100 µL from the 1 mL cell suspension (dilution factor of 1:10) was added into the new T75 flask pre-filled with 10 mL of respective media (see Recipe 3). For Huh-7 cells, 400 µL from the 1 mL cell suspension (dilution factor of 4:10) was added into the new T75 flask pre-filled with 10 mL of respective media (see Recipe 3). This allows the cells to reach confluency in 4–5 days.

2. In our study, we used Vero cells between passages 5 and 40, which have shown consistent stability and no unusual results. For Huh-7 cells, which are less stable at earlier passages, we have used them between passages 50 and 70, following their purchase at passage 46. No issues or peculiar results have been observed within this passage range.

D. Viruses and virus propagation

1. DENV-2, CHIKV, and ZIKV are propagated in Vero cells (Table 1).
2. Seed a monolayer of Vero cells in a T75 cell culture flask and allow the cells to reach 70%–80% confluency.
3. Thaw the virus master stock (either from earlier passage or newly purchased virus stock) at room temperature before use.
4. Add 5 mL of the diluted virus to the T75 flask containing the cell monolayer.
Note: The dilution factor depends on the virus concentration. Viruses with higher titer require a higher dilution factor.
5. Incubate the flask at 37 °C in a 5% CO_2 humidified atmosphere for at least 1 h to allow the virus master stock to absorb into the host cells.
6. After the absorption period, carefully aspirate the viral master stock inoculum from the flask using a serological pipette.
7. Replace the aspirated medium with 10 mL of fresh DMEM supplemented with 2% heat-inactivated FBS (see Recipe 4).
8. Depending on the viral strain, incubate the infected cells for the duration specified in Table 1.

Table 1. Information on virus propagation and titration

Virus	Strain	Host cells	Titration method	Incubation days
DENV-2	NGC (ATCC VR-1584)	Vero	Foci	4
CHIKV	ECSA (MY/065/08/ FN295485)	Vero	Plaque	2
ZIKV	P6740 (provided by UTMB)	Vero	Plaque	5

9. Once the host cells exhibit approximately 70% cytopathic effect (CPE), proceed to harvest the viral supernatant, which will be mentioned as working virus stock. Figure 3 shows DENV-infected Vero cells at four days post-infection, reaching 70% CPE and ready for virus harvest from the culture supernatant.

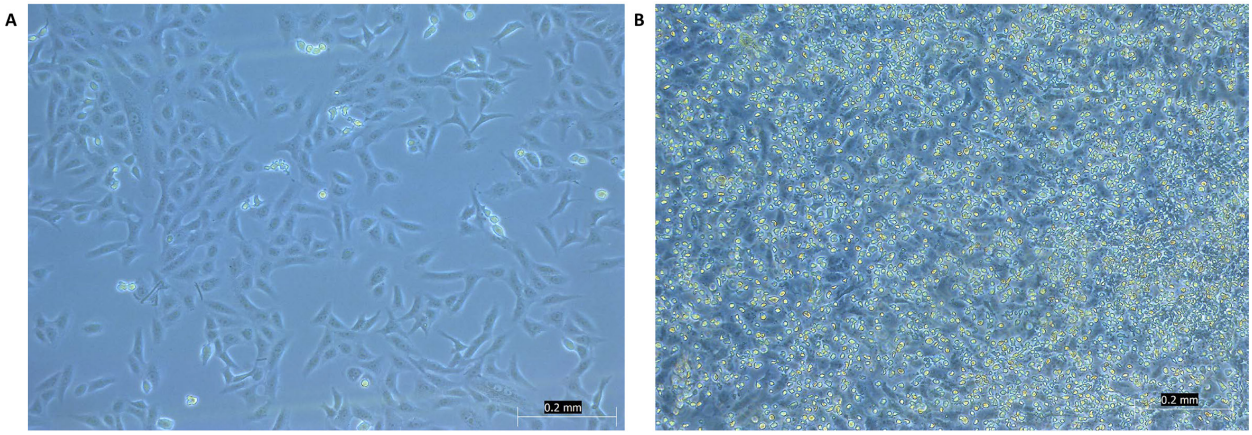


Figure 3. Morphology of Vero cells before and after dengue virus (DENV) infection. (A) Uninfected Vero cells. (B) DENV-infected Vero cells 4 days post-infection.

10. Carefully collect the supernatant and transfer it to a 15 mL tube.
11. Centrifuge the collected supernatant at 800× *g* for 10 min at 4 °C to remove cellular debris.

12. Transfer the clarified supernatant to labeled tubes by aliquoting 300 µL of viral stock per tube to minimize freeze-thaw cycles.
13. Store the aliquoted virus stock at -80 °C for long-term storage.
14. Perform all antiviral assays using the same batch of virus working stock to reduce variability in experimental results.

E. PBMC or pDC stimulation and PBMC conditioned media collection

1. Thaw the isolated PBMCs in a 37 °C water bath.
2. Transfer the thawed PBMCs into 10 mL of prewarmed PBMC culture media (see Recipe 1) in a sterile 15 mL tube.
3. Gently mix by inverting the tube 5–10 times.
4. Centrifuge the cells at 300× *g* for 3 min at room temperature.
5. Discard the supernatant.
6. Resuspend the PBMC pellet in an appropriate volume of PBMC culture media (see Recipe 1) to achieve a density of 8×10^5 cells/mL.
Note: If pDCs are intended for antiviral screening, freshly isolated pDCs should be counted and diluted to achieve a density of 8×10^5 cells/mL.
7. Transfer 250 µL of the resuspended PBMCs or pDCs (equivalent to 2×10^5 cells) into each well of a 96-well plate.
8. Prepare the potential antiviral compounds at the maximum non-toxic dose ($MNTD_{90}$) (see General note 2) and add 2 µL of the compound into each well containing 2×10^5 cells.
Note: Here, we demonstrate using oligodeoxynucleotides (ODNs) containing unmethylated cytosine-guanine dinucleotides (CpG) (CpG ODN 2216) as an example. Dilute 500 µM stock solution of CpG ODN 2216 to 125 µM and add 2 µL of 125 µM CpG ODN 2216 into the wells containing 2×10^5 cells in a total volume of 250 µL. This results in a final concentration of 1 µM CpG ODN 2216.

$$\frac{125\ \mu M \times 2\ \mu L}{250\ \mu L} = 1\ \mu M$$

9. Incubate the cells at 37 °C in a 5% CO_2 humidified atmosphere for 24 h.
10. After incubation, carefully harvest the stimulated cells together with the supernatant and centrifuge them at 300× *g* for 5 min at room temperature to pellet the cells.
11. Collect the conditioned media and aliquot 300 µL into multiple tubes to avoid freeze-thaw cycles.
12. Store the aliquots at -80 °C for long-term storage.
13. The stimulated cells are subjected to immunophenotyping via flow cytometry (Section F).
Note: Humoral factors in the conditioned media can be quantified using an ELISA assay or a high-throughput assay (HTA), such as the Bio-Plex Pro Human Cytokine 27-plex Assay.

F. Immunophenotyping via flow cytometry to gate and measure the activation of pDCs

1. After 24 h of treatment with the potential antiviral compounds (as described in section E), collect the stimulated PBMCs and pellet by centrifuging at 300× *g* for 5 min.
Note: Ensure all PBMCs are collected by observing under the microscope. If cells are not fully harvested, add 200 µL of staining buffer to each well and repeat the PBMC harvesting procedure twice.

2. Wash the cells with 10 mL of staining buffer and pellet them again by centrifuging at 300× *g* for 3 min.
3. While waiting, prepare an antibody cocktail based on Table 2. All antibodies are custom-made and purchased (refer to reagents 15–19).

Table 2. Antibody cocktail preparation

Surface markers	Florescence conjugate	Volume per reaction/μL
CD304	BB515	5
CD123	APC	5
CD86	PE	20
HLA-DR	PE-Cy7	5

4. After washing, resuspend the cell pellet in 200 μL of staining buffer and stain with 40 μL of antibody cocktail for 30 min on ice and away from a light source.
5. To remove unbound antibodies, wash the cells by adding 2 mL of staining buffer and centrifuge at 300× *g* for 3 min.
6. Resuspend the cell pellet in 300 μL of staining buffer, then add 20 μL of 7-AAD viability dye and incubate on ice for 5 min.
7. Finally, run the cells using the BD FACSCanto II flow cytometer and analyze data using FACSDiva software as shown in Figure 4.

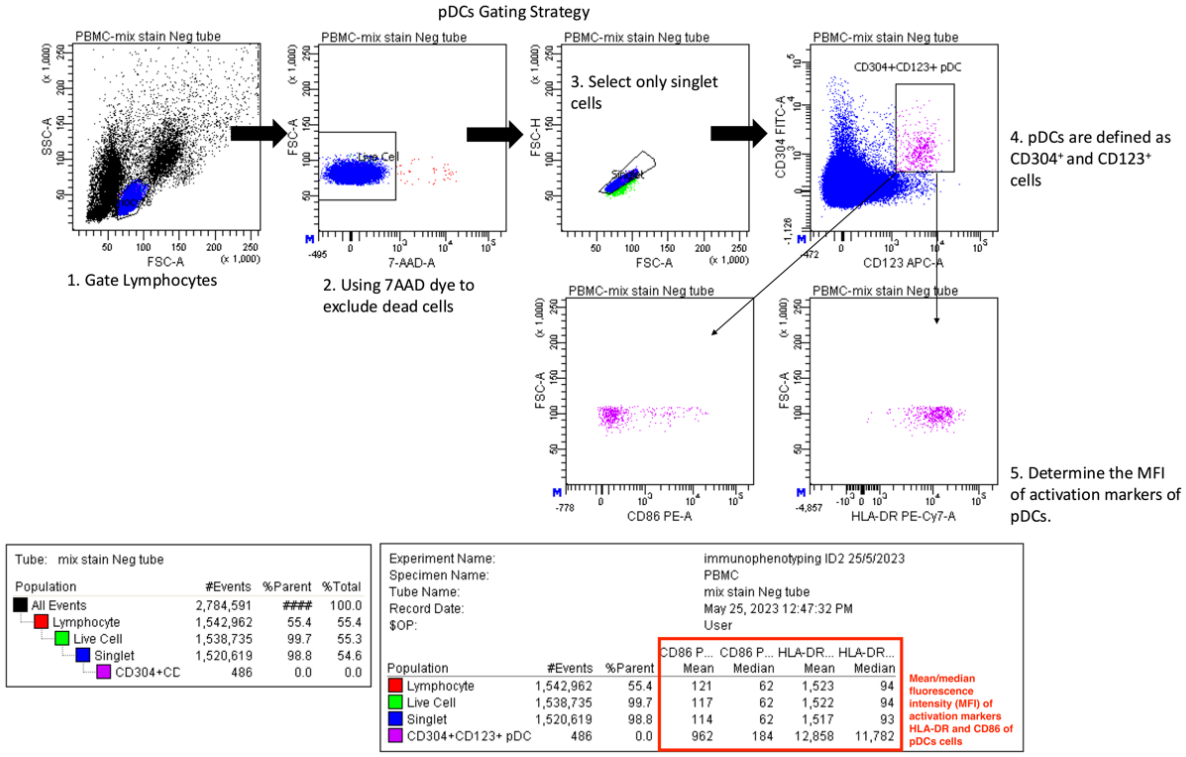


Figure 4. Gating strategy for pDCs and quantification of activation markers by flow cytometry. Representative flow cytometry plots illustrate the gating strategy used to identify pDCs within PBMCs, followed by the assessment of activation markers (HLA-DR and CD86) expression. The mean/median fluorescence intensity (MFI) of activation markers, HLA-DR and CD86, between the control and treated group can indicate the pDC activation.

Note: For flow cytometry, as long as the number of viable pDCs reaches at least 1,000 cells for evaluation, it is sufficient for the immunophenotyping assay. Since pDCs typically make up only 0.2%–0.8% of PBMCs, achieving this requires starting with at least 1.25 × 10^5 viable PBMCs. Therefore, maintaining the viability of PBMCs throughout the process is critical. In terms of viability thresholds, we recommend keeping the viability as high as possible, ideally above 80%, to ensure reliable gating and accurate results. If viability drops significantly below this level, it can lead to high levels of debris and nonspecific staining, which may compromise the accuracy of immunophenotyping. For preserving cells for later analysis, it is recommended to fix the PBMCs using 5% paraformaldehyde after the staining process. This helps to preserve cell morphology and immunophenotypic markers, allowing for reliable analysis at a later time.

G. Host cells stimulation by humoral factors derived from PBMCs or pDCs and antiviral assay

1. Pre-seed Huh-7 cells into 24-well plates at a density of 1 × 10^5 cells per well overnight in low glucose DMEM (see Recipes 3).
2. Prepare a 10-fold dilution of the conditioned media in cell culture maintenance media (see Recipe 4).
3. Remove the existing media from the Huh-7 cells and replace it with 1 mL of the 10-fold diluted immune cell-conditioned media.
4. Incubate the cells for 24 h at 37 °C in a humidified 5% CO_2 environment.
5. After incubation, carefully collect the conditioned media into a sterile 2 mL tube and infect the Huh-7 cells with the respective virus working stock at a multiplicity of infection (MOI) of 0.1 by adding 200 µL of the virus per well.

Note: While collecting and adding the 200 µL virus inoculum, ensure that the cells are not exposed to drying. Avoid removing the inoculum from multiple wells at once. Periodically observe the cells under a microscope to ensure the monolayer remains intact throughout the procedure.

6. Incubate the cells for 1 h at 37 °C to allow sufficient virus adsorption.
7. After the incubation, discard the unadsorbed virus and add back the 500 µL of the respective treatments (e.g., conditioned media or media from controls) into each well.
8. Incubate the cells for 48 h at 37 °C in a humidified 5% CO_2 environment.
9. Forty-eight hours post-infection, harvest the viral progeny from the cell culture supernatant by transferring it to labeled tubes.
10. Aliquot the viral supernatants appropriately to avoid repeated freeze-thaw cycles and store them at -80 °C.
11. Quantify the viral load in the supernatant using foci or plaque assays as detailed in sections H or I.

H. Quantification of infectious DENV using foci-forming unit assay (FFUA)

Note: The focus-forming unit assay (FFUA) is employed to titrate the unknown concentration of DENV stock, including both the virus working stock and the viral progeny obtained from antiviral assays.

1. Seed Vero cells in 24-well plates at a density of 1 × 10^5 cells/well and incubate overnight to form a monolayer.
2. Infect confluent Vero cells with ten-fold serial dilutions of DENV-2, starting with 10^{-1} dilution, by adding 200 µL of diluted virus per well.
3. Incubate for 1 h at 37 °C to allow virus adsorption.
4. Discard the unabsorbed virus suspension after the incubation period.
5. Overlay DENV-2-infected Vero cells with virus immobilizing media (see Recipe 2).
6. Incubate DENV-2-infected Vero cells at 37 °C for four days to allow the formation of countable foci.

7. After the incubation, remove the CMC overlay media from the wells using a Pasteur pipette.
8. Fix the cells with 500 µL of 5% paraformaldehyde at 4 °C for 1 h.
9. Discard the fixing solution and permeabilize the cells by adding 100-fold diluted Triton X-100 for 15 min at room temperature.
10. Rinse the cells with PBS three times after permeabilization.
11. Add 200 µL of the primary antibody (serum from DENV-recovered individuals) diluted in 3% skim milk at a ratio of 1:500.

Note: Primary antibodies can be sourced from the serum of DENV-recovered individuals, the 4G2 antibody that targets flaviviruses, or DENV-2-specific antibodies.

12. Incubate the cells with the primary antibody at 37 °C for 1 h.
13. Wash the cells three times with PBS to remove unbound primary antibodies.
14. Add 200 µL of the anti-human HRP-conjugated secondary antibody IgG (H + L) at a concentration of 1:1,000.

Note: The HRP-conjugated secondary antibody should be species-specific and designed to target the species in which the primary antibodies were generated. Since the primary antibody uses serum from DENV-recovered individuals, the secondary antibody should be specific to humans.

15. Incubate the cells with the secondary antibody at 37 °C for 1 h.
16. Wash the cells three times with PBS to remove unbound secondary antibodies.
17. Add 200 µL of TrueBlue peroxidase substrate in the dark and incubate for 15 min.
18. Remove the substrate by gently washing the cells using distilled water and air dry the plate.
19. Count the foci in each well and determine the viral titer as foci-forming units per milliliter (FFU/mL) (Equation 1, Figure 5).
20. Determine the antiviral effect by comparing the virus titer between the control and treatment groups by calculating the number of log reduction (Equation 2) or percentage of inhibition (Equation 3).

$$\text{Virus Titer}\left(\frac{\text{FFU or PFU}}{\text{mL}}\right) = \frac{\text{Average Number of Foci or Plaque (FFU or PFU)}}{\text{volume (mL)} \times \text{dilution factor}} \quad \text{(Eq. 1)}$$

$$\text{No. of Log Reduction} = \log_{10}\frac{\text{Virus Titer (Control)}}{\text{Virus Titer (Treatment)}} \quad \text{(Eq. 2)}$$

$$\text{Percentage of Inhibition (\%)} = \left(\frac{\text{Virus Titer Control} - \text{Treatment}}{\text{Virus Titer Control}}\right) \times 100\% \quad \text{(Eq. 3)}$$

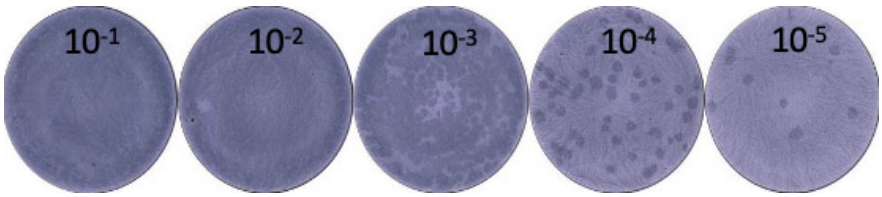


Figure 5. Example of dengue virus (DENV) titration and staining using the foci-forming unit assay (FFUA). 10^{-1} represents a 1×10^{-1} dilution, indicating a 10-fold dilution of the unknown virus titer supernatant. Similarly, 10^{-2} represents a 1×10^{-2} dilution, indicating a 100-fold dilution of the unknown virus titer supernatant, and so on for subsequent dilutions.

I. Quantification of infectious CHIKV and ZIKV using plaque forming unit assay (PFUA)

Note: The PFUA is employed to titrate the unknown concentration of CHIKV and ZIKV stock, including both the virus working stock and the viral progeny obtained from antiviral assays.

1. Seed Vero cells in 24-well plates at a density of 1×10^5 cells/well and incubated overnight to form a monolayer.
2. Infect confluent Vero cells with ten-fold serial dilutions of CHIKV and ZIKV by adding 200 µL of diluted virus per well.
3. Incubate for 1 h at 37 °C to allow virus adsorption.
4. Discard the unabsorbed virus suspension after the incubation period.
5. Overlay CHIKV-infected Vero cells and ZIKV-infected Vero cells with immobilizing media (see Recipe 2).
6. Incubate infected Vero cells at 37 °C for 2 days for CHIKV and 5 days for ZIKV to allow the formation of countable plaque.
7. After the incubation, remove the CMC overlay media from the wells using a Pasteur pipette.
8. Fix the cells with 500 µL of 5% paraformaldehyde at 4 °C for 1 h.
9. Remove the paraformaldehyde and stain the cells with 200 µL of 0.5% crystal violet for at least 10 min.
10. Gently wash the cells using distilled water and air dry the plate.
11. Count the foci in each well and determine the viral titer as plaque-forming units per milliliter (PFU/mL) (Equation 1, Figure 6).
12. Determine the antiviral effect by comparing the virus titer between the control and treatment groups by calculating the number log reduction (Equation 2) and percentage of inhibition (Equation 3) (Table S1).

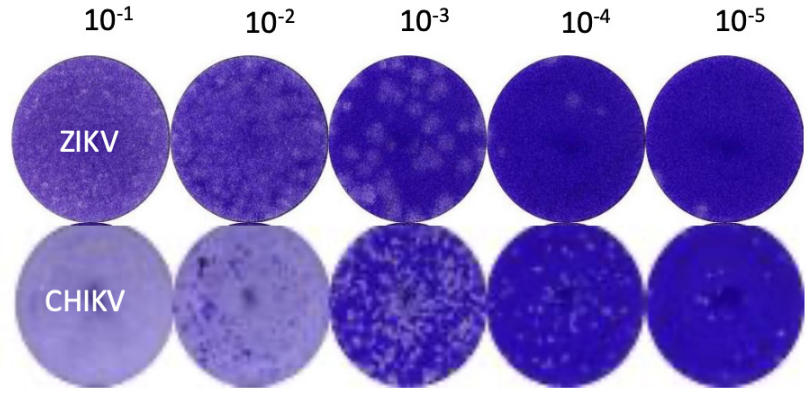


Figure 6. Example of Zika virus (ZIKV) (upper row) and chikungunya virus (CHIKV) (lower row) titration and staining using the plaque forming unit assay (PFUA). 10^{-1} represents a 1×10^{-1} dilution, indicating a 10-fold dilution of the unknown virus titer supernatant. Similarly, 10^{-2} represents a 1×10^{-2} dilution, indicating a 100-fold dilution of the unknown virus titer supernatant, and so on for subsequent dilutions.

J. Gene expression study

1. Pre-seed Huh-7 cells into 24-well plates at a density of 1×10^5 cells per well overnight.
2. Prepare a 10-fold dilution of the immune cell-conditioned media in low-glucose DMEM, ensuring each medium is supplemented with 2% FBS.
3. Remove the existing media from the Huh-7 cells and replace it with 1 mL of the 10-fold diluted immune cell-conditioned media.

4. Incubate the cells for 24 h at 37 °C in a humidified 5% CO_2 environment.
5. Next, discard the immune cell-conditioned media and extract total cellular RNA using the RNeasy kit as detailed in section K. Convert the RNA to cDNA using the iScript cDNA Synthesis kit as described in section L, and analyze gene expression with Luna Universal qPCR master mix according to section M.

K. Total cellular RNA extraction

1. The RNeasy kit is recommended to extract whole-cell RNA.
2. After 24 h of Huh-7 cell stimulation (as described in section J), discard the cell culture supernatant.
3. Add 350 µL of RTL buffer (from RNeasy Kit) to each well containing conditioned media-stimulated adherent cells.
Note: For adequate RNA yield (>100 µL) for gene expression analysis, use a total of 4 × 10^5 cells (equivalent to 4 wells of 1 × 10^5 cells). To lyse all cells, transfer the 350 µL of RTL buffer sequentially from one well to the next.
4. Homogenize the cell lysate by passing it through a 20-gauge (0.9 mm) needle attached to a sterile plastic syringe 5–10 times.
5. Vortex the cell lysate and briefly centrifuge to bring down any remaining lysate.
6. Add 350 µL of 70% ethanol to the lysate.
7. Carefully transfer 700 µL of the cell lysate onto the RNeasy spin column, avoiding touching the rim of the column.
8. Centrifuge at 8,000× *g* for 15 s and discard the flowthrough.
9. Add 700 µL of RW1 buffer to the spin column to remove contaminants.
10. Centrifuge at 8,000× *g* for 15 s and discard the flowthrough.
11. Add 500 µL of RPE buffer to the spin column.
12. Centrifuge at 8,000× *g* for 15 s and discard the flowthrough.
13. Add 500 µL of RPE buffer to the spin column.
14. Centrifuge at 8,000× *g* for 2 min and discard the flowthrough.
15. Add 30 µL of RNase-free water directly onto the membrane of the RNeasy spin column.
Critical: Avoid adding RNase-free water to the sides of the column, as it may not fully saturate the membrane and could lead to incomplete RNA elution. Ensure no water is trapped at the rim.
16. Measure the RNA concentration using a NanoDrop™ 2000/2000c spectrophotometer.
17. Verify that the RNA concentration is above 100 µg/µL and the A260/A280 purity ratio is between 1.8 and 2.0.

L. cDNA synthesis by reverse transcription (RT) reaction

1. Use the iScript cDNA Synthesis Kit to convert total RNA into cDNA, following the manufacturer's protocol.
2. Dilute the total RNA extracted from Huh-7 cells (see section K) to a concentration of 60 ng/µL (for a final quantity of 1.2 µg in a 20 µL cDNA synthesis reaction) with RNase-free water. Keep the diluted RNA on ice until the next step.
3. Prepare a cDNA synthesis master mix by combining 4 µL of 5× iScript Reaction Mix, 1 µL of iScript Reverse Transcriptase, and 10 µL of RNase-free water (Table 3).
4. Aliquot 15 µL of the cDNA synthesis master mix into each PCR tube.

Note: The total volume of cDNA synthesis master mix depends on the number of samples and reactions needed, with a minimum of two reactions (e.g., treatment and control groups). It is recommended to prepare an extra reaction to ensure sufficient volume. The table below provides an example of preparing for three reactions.

Table 3. Volume of each component in the cDNA synthesis using the iScript cDNA Synthesis kit

Component	Volume (µL) per reaction	Volume (µL) per 3 reactions
5× iScript Reaction Mix	4	12
iScript Reverse Transcriptase	1	3
RNase-free water	10	30

5. Add 5 µL of diluted RNA (60 ng/µL) to each PCR tube containing 15 µL of the cDNA synthesis master mix.
6. Incubate the mixture in the Applied Biosystems™ SimpliAmp™ thermal cycler under the following conditions: 25 ˚C for 5 min to allow priming, 46 ˚C for 20 min to facilitate reverse transcription, and 95 ˚C for 1 min to inactivate the cDNA synthesis reaction (Table 4).

Table 4. Thermocycling conditions for the cDNA synthesis using iScript cDNA Synthesis kit

Step	Temp. (˚C)	Duration	Cycle
Priming	25	5 min	1
Reverse transcription	46	20 min	1
Reverse transcription inactivation	95	1 min	1
Hold	4	∞	1

M. ISGs expression study via quantitative real-time polymerase chain reaction (qRT-PCR)

1. Relative qRT-PCR is performed to quantify the expression of ISGs using Luna Universal qPCR master mix and the QuantStudio 5 Real-Time PCR System.
Note: The primer sequence can be adjusted to evaluate different target genes or alternative housekeeping genes. The expression of ISGs is recommended in this protocol, with primer sequences adopted from a previously published study [1].
2. Prepare a qRT-PCR master mix according to Table 5.
Note: For example, if you have two samples and require five PCR reactions (two reactions per sample in duplicate, plus an extra reaction), adjust the master mix volume accordingly. If using multiple primer sets to assess various gene expressions, prepare separate qRT-PCR master mixes for each primer set.

Table 5. Volume of each component in the qPCR reaction using Luna Universal qPCR master mix

Component	Volume (µL) per reaction	Volume (µL) per 5 reactions
Luna Universal qPCR mix	10	50
Forward primer (10 µM) (refer to Table 6)	0.5	2.5
Reverse primer (10 µM) (refer to Table 6)	0.5	2.5
RNase-free water	8	40

Table 6. Primer sequences for amplification and fold-change quantification of IFN-stimulating genes (ISGs). These primer sequences are adapted from a previous publication by Tsuji et al. [1].

ISGs targets	Forward Primer sequence	Reverse Primer sequence
ACTB	tggcattgccgacaggatgcag	gcatttgcggtggacgatggag
RyDEN	cgcatgtttcaacgtgcccagga	tgagtgtgggtgcggaagttgtgc
IFITM-1	ccgaccatgtcgtctggtccctgtt	tgttcaggcacttggcggtgga
OAS-1	atcgccggggagagttcatcca	agggcatcaaaggcaggcagca
ISG15	tggcgggcaacgaattccagg	tcgcatttgtccaccaccagca
ISG20	tggctcgttgcagcctcgtgaa	tctagcctggccacggcaaatggt
RSAD2	tgctgccccttgaggaagcaaa	tcccggatcaggcttccattgc
Mx-A	tccgtgttggaggcactgtcagga	cggcgatggcattctgggcttt

3. Aliquot 19 µL of the qRT-PCR master mix into each well of the PCR strip tubes.
4. Add 1 µL of the cDNA template onto the wall of each PCR strip tube.
5. Centrifuge the tubes to bring the cDNA template down to the master mix.
6. Briefly vortex the PCR mixture, then centrifuge again to ensure the qRT-PCR reaction components are collected at the bottom.
7. The qRT-PCR reaction is initially denatured at 95 °C for 1 min, then amplified by 40 cycles at 95 °C for 15 s (denaturation) and 60 °C for 1 min (annealing) and 72 °C for 30 s (extension) using QuantStudio 5 real-time PCR system (Table 7).

Table 7. Thermocycling conditions for the qPCR reaction using Luna Universal qPCR master mix

Step	Temp. (°C)	Duration	Cycle
Initial denaturation	95	1 min	1
Denaturation	95	15 s	
Annealing	60	1 min	30
Extension	72	30 s	
	95	15 s	
Melt curve	60	1 min	∞
	95	1 s	

8. Determine the fold change in ISGs (Table S2) using Design & Analysis 1 software v1.5.2, compatible with the QuantStudio 5 system.
9. A step-by-step guide to set up the Design & Analysis 1 software v1.5.2 is shown in Figure 7.

Note: The RQ value of the untreated sample (negative control) is used as the reference, with an RQ value set to 1.

Figure 7. Ten-step guide for using the Design and Analysis (DA1) software. Comprehensive instructions for setting up the experimental template, performing automatic normalization with an endogenous control (housekeeping gene), and automatically calculating the relative fold change in gene expression using a reference sample (e.g., untreated or negative control). (A) Set up the experiment type as a comparative Ct. (B) Set up the qPCR run method as indicated in Table 6. (C) Set up the targets (gene of interest) and samples (various treatment groups). (D) Set up reference dye, endogenous control (housekeeping gene), and reference sample (untreated control). (E) Save the electronic data template (EDT) file into a drive. (F) Load the prepared PCR mix and run the EDT file in QuantStudio 5 real-time PCR system. (G) After the qPCR run, save the electronic data sheet (EDS) file back into the drive. (H) Open the EDS file in Design & Analysis 1 software v1.5.2. (I) Evaluate the melt curve plot to ensure the melt curve is a single peak and the Tm is consistent for each gene. (J) Export the results as an Excel file. The relative quantification (RQ) value is the relative gene expression of the gene after treatment.

Data analysis

1. Perform statistical analyses using the EZR software [18].
2. Conduct a normality test using the Kolmogorov–Smirnov normality test.
3. If the dataset does not meet the assumptions of normality, or if the sample size is small, a non-parametric approach should be used.
4. Use the Kruskal–Wallis test to assess significant differences among groups and conduct the Steel test for subsequent multiple comparisons between groups.
5. Present data using median ± standard deviation (SD).
6. Define statistical significance at $p < 0.05$ and report data with a 95% confidence interval.

Validation of protocol

This protocol or parts of it has been used and validated in the following research article(s):
• Tsuji et al. [1]. Induction of anti-viral genes mediated by humoral factors upon stimulation with Lactococcus lactis strain plasma results in repression of dengue virus replication in vitro. *Antiviral Research* (Figure 1 and Figure 2).
• Ishii et al. [17]. Plasmacytoid dendritic cells stimulated with Lactococcus lactis strain Plasma produce soluble factors to suppress SARS-CoV-2 replication. *Biochemical and Biophysical Research Communication (BBRC)* (Figure 2B)
• Low et al. [19]. The antiviral effects of heat-killed Lactococcus Lactis Strain Plasma against Dengue, Chikungunya, and Zika Viruses in human by upregulating the IFN-α signaling pathway. *Microorganisms* (Figures 2, 3, and 4).

General notes and troubleshooting

General notes

1. Using human immune cells such as PBMCs or pDCs for antiviral screening offers a significant advantage: the potential to identify novel, broad-spectrum antivirals that target host cell mechanisms while reducing the risk of resistance and uncovering antiviral activities that may not be detected with conventional cell line–based assays.
2. We recommend verifying the cytotoxicity of potential antiviral compounds in PBMCs or pDCs before progressing to Section E of the protocol. The maximum non-toxic dose ($MNTD_{90}$) can be determined using an MTS assay, where cells are treated with serial dilutions of the antiviral compounds for 24 h.

3. Sample size should be calculated and clearly defined to ensure the study has adequate power to detect a meaningful effect and enhance its validity. This calculation can be performed using software like G*Power, PASS, or nQuery or through online calculators. It is crucial to carefully consider all parameters involved in the sample size calculation to ensure accurate and reliable results. For example:
a. Power (1-β): The probability of detecting a true effect (commonly set at 80% or 90%).
b. Significance level (α): The probability of a Type I error, usually set at 0.05.
c. Effect size: The expected difference between groups may be based on prior studies or clinical significance.
d. Variability (standard deviation): A measure of the data's spread, often derived from previous studies.
e. Type of study design: For example, parallel, crossover, or factorial designs affect the sample size calculation.

4. This protocol is designed for screening antivirals with the potential for systemic administration, either through direct injection into the bloodstream or absorption via the mucosal layer following oral intake. We particularly recommend screening repurposed drugs, as they offer several advantages, including established safety profiles and a significantly faster development timeline compared to novel compounds. Repurposed drugs also have well-documented pharmacokinetics and toxicity data, making them promising candidates for rapid deployment in antiviral therapy.
5. This protocol can be adapted for screening antivirals targeting a wide range of viruses not limited to DENV,

CHIKV, and ZIKV: also, other virus families such as Influenza A virus subtype H1N1, respiratory syncytial virus (RSV), and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).

Troubleshooting

Problem 1: Low pDC count after isolation or low pDC count in the PBMC population during immunophenotyping assays.
Possible cause: Insufficient volume of whole blood collected during the blood drawing procedure.
Solution: Increase the blood volume collected, subject to approval from the ethics committee. Consider pooling samples by combining PBMCs or pDCs from multiple donors' post-stimulation.

Note: Culturing pooled PBMCs or pDCs is not recommended because genetic variations among individuals may activate immune cells, causing false-positive results. If pooling samples is necessary as a last resort, we recommend stimulating PBMCs or pDCs separately and then pooling them only for flow cytometry analysis. Optimization is critical to determine the appropriate number of pDCs or PBMCs needed for effective antiviral screening and the total blood volume required from each participant.

Supplementary information

The following supporting information can be downloaded here:
1. Table S1. The antiviral effect of recombinant IFN-α (100U), LCP Sup 1:10, and CpG Sup 1:10, were evaluated based on the log reduction normalized to Neg Sup 1:10 (median ± SD) N = 5
2. Table S2. Fold-increase in ISGs of Huh-7 cells following treatments with recombinant IFN-α (100 units), CpG Sup 1:10, and LCP Sup 1:10, (median ± SD) N = 5.

Acknowledgments

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Competing interests

The authors declare that this study received funding from the Institute of Health Sciences, Kirin Holdings Co., Ltd. The funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article, or the decision to submit it for publication.

Ethical considerations

The study was conducted in accordance with the Declaration of Helsinki, and approved by The Medical Research Ethics Committee of Universiti Malaya Medical Centre on 6 May 2022 (UMMC MREC No. 202256-11216). Informed consent was obtained from all subjects involved in the study.

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