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Functional genomics approach for the identification of human host factors supporting dengue viral propagation

Nicholas J. Barrows^{1,2,3,6}, Sharon F. Jamison^{1,2}, Shelton S. Bradrick^{1,2}, Caroline Le Sommer^{2,‡}, So Young Kim^{1,2,6}, James Pearson^{2,6,π}, and Mariano A. Garcia-Blanco^{1,2,5,¶}

¹Center for RNA Biology, Duke University Medical Center, Durham, NC 27710, USA

²Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, NC 27710, USA

³Program in Cell and Molecular Biology, Duke University Medical Center, Durham, NC 27710, USA

⁵Department of Medicine, Duke University Medical Center, Durham, NC 27710, USA

⁶Duke RNAi Screening Facility, Duke University Medical Center, Durham, NC 27710, USA

Abstract

Dengue virus (DENV) is endemic throughout tropical regions around the world and there are no approved treatments or anti-transmission agents currently available. Consequently, there exists an enormous unmet need to treat the human diseases caused by DENV and block viral transmission by the mosquito vector. RNAi screening represents an efficient method to expand the pool of known host factors that could become viable targets for treatments or provide rationale to consider available drugs as anti-DENV treatments. We developed a high throughput siRNA-based screening protocol that can identify human DENV host factors. The protocol herein describes the materials and the procedures necessary to screen a human cell line in order to identify genes which are either necessary for or restrict DENV propagation at any stage in the viral life cycle.

Keywords

RNA interference (RNAi); dengue virus; yellow fever virus; whole genome RNAi screening; whole genome siRNA screening; dengue virus host factors; flavivirus

1. Introduction

Dengue virus (DENV) is endemic throughout tropical regions around the world as is its primary mosquito vector, *Aedes aegypti*. The number of countries reporting dengue-related diseases to the World Health Organization increased at a rate of 13 countries per decade from 1955 to 2004, demonstrating an emerging threat to populations worldwide [1].

[¶]Send correspondence to M. A. Garcia-Blanco, m.garciablanca@duke.edu, Box 3053 (0036 CARL), Duke University Medical Center, 213 Research Drive, Durham, NC 27710 USA; Tel 919-613-8632 (direct) Fax 919-613-8646.

[‡]Current address The Hamner Institutes for Health Sciences, Research Triangle Park, NC 27709, USA

^πCurrent address Bayer Crop Sciences, Research Triangle Park, NC, 27709 USA

Currently, there is no effective vaccine that can protect against all four DENV serotypes and only palliative care is available for infected patients suffering from severe disease. The only proven way to combat urban outbreaks requires a combination of public health measures, including promotion of mosquito control and personal protection [2]. Consequently, there exists an enormous unmet need to treat the human diseases caused by DENV and block viral transmission by the mosquito vector. Rapid identification of host factors and biological processes that either promote or restrict virus propagation may advance development of anti-viral therapeutics. The recent utilization of whole-genome RNA interference (RNAi) screens identified hundreds of human and insect host factors that are required for efficient DENV propagation [3,4]. RNAi is a cellular process in which small interfering RNAs (siRNA) bind perfectly complementary mRNA targets and induce their endonucleolytic cleavage [5,6]. The consequence of RNAi is the depletion of the targeted mRNA and their protein products. Genome-scale RNAi uses either chemically synthesized siRNA duplexes or expressed short hairpin RNAs (shRNA) to target mRNAs. These libraries of siRNAs and shRNAs are used to systematically evaluate the roles that genes play in various biological processes [7]. RNAi screening represents an efficient method to expand the pool of known host factors that could become viable targets for treatments or provide rationale to consider available drugs as anti-DENV treatments.

We developed a high throughput siRNA-based screen to identify human DENV host factors. Our strategy used two independent siRNA libraries which are simultaneously tested. Host factor candidates are identified only when the two independent tests provide mutually supportive results (*see* Note 1) [8,9]. The protocol herein describes the materials and the procedures used to screen 2,240 genes or one batch of siRNA containing plates. An entire genomic screen is divided into eleven nearly-equal batches. Reverse transfection of siRNAs into cells, infection of the transfected cells with DENV, and fixation and staining of the infected cells are detailed (*see* Note 2 and Note 4).

¹Ideally, each siRNA treatment leads to profound depletion of the host protein(s) encoded by the targeted transcripts and does not affect the levels of any other protein in the cell. Practically, however, siRNAs exhibit variable potency against their intended target mRNAs and can down regulate expression of unintended ones – so called off-target effects [11]. As such, the standard of the field requires that any RNAi-based result is deemed reliable only when multiple independent siRNAs provide evidence that supports the hypothesis. Moreover, it is desirable that RNAi-mediated phenotypes be rescued by re-expression of a siRNA-resistant mRNA that encodes the protein of interest, and additionally it is judicious to confirm RNAi phenotypes by other genetic or biochemical methods. At the completion of a genome-scale screen using an RNAi library design similar to the design we employ, the screener(s) generate a hit list in which each hit has at least two siRNAs that produce the interrogated phenotype.

²We always test our proposed procedures prior to beginning any large scale screen. First, the entire written procedure is applied using a single microtiter assay plate from the siRNA library. The outcome of this practice is to determine if there exists a significant error in the written or planned procedure which would lead to failure to produce high quality data. We recommend that unexpected observations made during this test are explored prior to moving forward with a pilot screen. Second, the procedure is tested against a set of microtiter assay plates during a pilot screen. The goal of the pilot screen is to determine if there is any reason not to move forward with the complete screen. The pilot screen represents the last opportunity to identify problems or reconsider the experimental design. The ideal pilot screen assay plates permit the screening team to complete every step of the procedure for a single batch of microtiter assay plates including limited data analysis with the intention of producing a short 'hit' list. Further, the same set of microtiter assay plates will be tested again during the screen and the data from the pilot screen and genomic screen can be compared directly. It is expected that the results from individual wells will behave similarly between the pilot and genomic screen plates which demonstrates that the protocol is applied effectively.

⁴The number of microtiter assay plates which are prepared each day is dependent on how many plates can be efficiently processed by the screening team and dependent on the capacity to image the plates before the next batch is ready the following day. We chose to process 14 microtiter assay plates each day. Therefore, there are 11 batches for an entire genomic screen with two separate libraries formatted on a total of 144 plates. We arranged the screening schedule such that a new batch was started each day so the entire screen takes less than 3 weeks from initiating cell culture to producing the first genomic database of screening data.

2. Materials

2.1. Tissue culture, siRNA transfection and virus infection

1. Tissue culture medium: DMEM, 10% FBS, PS 100U/mL
 1. Dulbecco's modified Eagle medium (DMEM).
 2. . Penicillin-Streptomycin (PS), 10,000 U/mL
 3. Heat-inactivated fetal bovine serum (FBS)
2. Complete screening medium: DMEM, 5% FBS, 0.01M HEPES, PS 100U/mL
 1. Dulbecco's modified Eagle medium (DMEM)
 2. . Penicillin-Streptomycin (PS), 10,000 U/mL
 3. Heat-inactivated fetal bovine serum (FBS)
 4. HEPES, 1M Buffer Solution
3. Phosphate buffered saline without calcium or magnesium (PBS)
4. Trypsin/EDTA, 0.25%
5. Opti-MEM® Reduced Serum Medium, no Phenol Red (Opti-MEM) (Gibco# 11058-021)
6. HuH-7 cells (*see* Note 11)
7. Qiagen siRNA duplex targeting green fluorescent protein (Qiagen# SI04380467)
8. Qiagen siRNA duplex targeting ATP6V0C (ATP6V0C siRNA) (Qiagen# SI00307384)
9. Qiagen AllStars Negative Control siRNA (Qiagen # 1027280)
10. Qiagen Human Whole Genome siRNA set, Library v1.0 (*see* Note 5)
11. 384 Well Flat Clear Bottom Black Polystyrene TC-Treated Microplates (microtiter assay plate) (Corning# 3712)
12. 75cm² tissue culture flasks, vented caps (T-75 flask)

¹¹Two qualities possessed by HuH-7 cells makes the cell line an ideal choice for siRNA screens[12]. First, the HuH-7 cell line is susceptible to DENV infection and produces infectious progeny virus; essential to our assay, which evaluates all stages in viral lifecycle. Second, HuH-7 cells grow as an evenly distributed monolayer with well-separated and regularly ovoid nuclei facilitating automated imaging and analysis.

⁵Our protocol applied the Qiagen Human Whole Genome siRNA set, Library v1.0 towards interrogating 22,909 predicted mRNAs in which each mRNA is targeted by four unique chemically synthesized siRNA duplexes labeled A, B, C or D. Ideally, each of the four siRNAs target a unique, non-overlapping sequence which does not have homology with a different gene, however this is not always the case. The siRNAs are arrayed in columns 3–22 and rows A–P of the microtiter assay plate. Each well contains 1pmol total siRNA in 5µL dH₂O. The siRNAs are arrayed prior to the project and stored at –80°C. The final concentration for the siRNAs, after addition of Opti-MEM, RNAimax and cell suspension, is 15.4nM. Our library format uses a 2x2 pooled arrangement such that siRNA duplexes A and B targeting gene X are arrayed in one well of a 384 well microtiter assay plate while siRNA duplexes C and D targeting gene X are arrayed on a separate 384 well microtiter assay plate. Both siRNA pools are located at analogous positions within each paired 384 well microtiter assay plate creating two independent libraries which differ only by siRNA sequence identity. The AB and CD libraries are screened and high confidence targets are identified when positive in both libraries thereby limiting false-positive results incurred by testing a single pool of siRNAs multiple times[8]. Indeed siRNA induced phenotypes are very precise and repetition of single pools of siRNAs is unlikely to improve on accuracy. While our approach minimizes false-positives, one caveat associated with this strategy is that it likely elevates the rate of false-negatives due to ineffective siRNA sequences.

13. Lipofectamine RNAiMax (Invitrogen# 13778-150)
14. Corning® 125mL Octagonal PET Storage Bottles, Sterile (Corning # 431731)
15. Corning® 500mL Octagonal PET Storage Bottles, Sterile (Corning # 431733)
16. Dengue 2 virus, New Guinea C strain (*see* Note 14)
17. 384ST 70µl Tips, Sterile, (ST70 tips) (Agilent Technologies # 19133-012)
18. 1mL, 5mL, 10mL, 25mL and 50mL serological pipettes
19. Omni Trays (Thermo # 242811)
20. Matrix WellMate Tubing Assemblies (Matrix cartridge) (Thermo # 201-30002)
21. Thermo Scientific Matrix WellMate Flexible, High-Speed, 8-Channel Microplate Dispenser
22. Rainin manual multichannel pipette (Rainin# L12-20XLS; P20)
23. Sorvall Legend RT+ centrifuge with Sorvall S1102 swinging bucket rotor

2.2. Fixation and viral envelope protein staining

1. 4% Paraformaldehyde (w/v) (4% PFA):
 1. Make at least 3300mL 4% PFA diluted in PBS immediately prior to the genomic screen. Filter through a 0.45µm filter to remove particulates which disrupt image analysis, and store at 4°C.
2. 4500mL PBS with 0.1% Tween-20 (PBS-tween)
 1. Add 450mL 10x Dulbecco's Phosphate Buffered Saline to 4050mL 0.45µm filtered H₂O. Filtering is necessary to remove particulates which disrupt image analysis.
 2. Add 4.5mL Tween-20
 3. Mix well
3. 10x Dulbecco's Phosphate Buffered Saline
4. Tween-20, Sigma# P1370-100ML
5. bisBenzamide H 33342 trihydrochloride (Hoechst)
 1. Dilute 10mg/mL in dH₂O, store at 4°C protected from light
6. Triton X-100
7. Pan-flavivirus anti-DENV envelop protein mouse monoclonal antibody (4G2)[10]
8. Goat anti-mouse AlexaFluor 488 (Alexa488)
9. Normal Donkey Serum (Millipore# S30-100mL)

¹⁴The HuH-7 cells were infected with MOI 0.2 using dengue 2, New Guinea C strain viral stock produced by the *Aedes albopictus* derived C6/36 cell line. The viral titer is determined by infection of Vero (African green monkey kidney) cell monolayers followed by quantification by a foci formation assay.

10. Bottle top Vacuum filter, 1L
11. Rainin LTS pipette tips 20 μ L
12. Rainin LTS pipette tips 200 μ L
13. Matrix pipette tips 1250 μ L (Matrix# 8042)
14. Adhesive plate sealing film (Fisher# AB-0580)
15. Reagent reservoirs
16. Ethanol 70%
17. Ethanol 100%
18. Distilled water
19. Kimtech Science Kimwipes
20. Velocity11/Agilent Bravo Liquid Handling Platform
21. Biotek ELx405 96 well plate washer
22. Cellomics Target Activation software package and associated ArrayScan^{VTI} instrumentation and robotic arm
23. VP179 aspiration manifold, V&P Scientific
24. Matrix Impact2 Multichannel Electronic Pipette
25. Baker BioPROtect II biological safety cabinet

3. Methods

3.1. Tissue culture, Day 0

1. Prepare five T-75 flasks each with 2.0×10^6 HuH-7 cells in 14 mL tissue culture medium (*see* Note 3)

3.2. Reverse transfection, Day 2 Time started: _____

1. Remove 14 microtiter assay plates from the -80°C freezer (*see* Note 4 and Note 5). Set on the bench to defrost at room temperature. Record microtiter Assay Plate ID labels.
2. Warm the incomplete screening medium (DMEM, 5% FBS, PS), 0.25% Trypsin, PBS, Opti-MEM, 1M HEPES in water bath set to 37°C . The HEPES is added to the incomplete screening medium during subsequent steps to create the complete screening medium.
3. Sanitize the laminar flow cabinet with 70% ethanol.

³Three T-75 flasks of HuH-7 cells are used for the siRNA reverse transfection each day. Two T-75 flasks of HuH-7 cells are used for tissue culture in order to generate the next five T-75 flasks of HuH-7 cells needed for another batch of microtiter assay plates. In order to avoid passaging the same cells every day, we set up parallel cell passages. Passage A was used to start screens on days even numbered days while Passage B was used to start screens on odd numbered days.

4. Aliquot 200mL 70% ethanol into a T-75 flask. Retrieve the Matrix cartridge (*see* Note 6). Run ethanol through the Matrix cartridge in order to sanitize it. Let sit until needed with ethanol in the line.
5. When microtiter assay plates are fully defrosted, centrifuge the plates at room temperature at 500g for 1 minute in a centrifuge with swinging bucket rotor. Transfer plates to the laminar flow cabinet.
6. Sanitize the outside of the microtiter assay plates with 70% ethanol.
7. Ensure that there is at least 300µL of 0.2µM ATP6V0C siRNA and 0.2µM AllStars Negative Control siRNA solutions (*see* Note 7).
8. Remove the plate seals from all microtiter assay plates carefully.
9. For each microtiter assay plate, use the P20 multichannel pipette to:
 1. Remove all dH₂O from wells E2, G2, I2, K2 & F23, H23, J23, L23.
 2. Add 5µL 0.2µM ATP6V0C siRNA to wells E2, G2, J23, L23.
 3. Add 5µL 0.2µM AllStars Negative Control siRNA to wells I2, K2, F23, H23.
 4. Any remaining siRNA can be frozen and used the following day.
10. Centrifuge the microtiter assay plates at room temperature at 500 x g for 1 minute in order to make sure the control siRNAs are concentrated at the bottom of the well.

⁶Consistent implementation of the screening protocol maximizes the distinction between a negative control population and valuable hits. Our group utilizes several useful technologies which facilitate screening.

1. The Thermo Scientific Matrix WellMate Flexible, High-Speed, 8-Channel Microplate Dispenser evenly dispensed the transfection reagent and HuH-7 cells into the microtiter assay plates.
2. The consistent distribution of DENV-containing media within wells on each plate, between plates, and across several days in a screen is essential in order to identify wells with low infection. The Velocity11/Agilent Bravo Liquid Handling Platform delivers and gently mixes viral-containing media in the 384 microwell plate.
3. Incomplete microwell washing could prevent identification of interesting hits. The wash steps during immunofluorescence staining procedure must be performed consistently so that the fluorescent signal from each well accurately reflects the DENV infection within the well. The Biotek ELx405 96 well plate washer is used to perform all wash steps.
4. Finally, the Cellomics Target Activation software package is applied to images gathered by the ArrayScan ^{VTI}. While the liquid handling apparatus improve consistent application of the protocol, the imaging and analysis of the well directly determines which statistics will contribute to the final analysis, therefore great care must be taken when developing the computation portion of the project.

⁷The siRNA duplex targeting green fluorescent protein (GFP siRNA) and AllStars Negative Control siRNA served as the negative controls while siRNA duplex targeting the vacuolar ATPase served as the positive control during assay development and RNAi screening. The vacuolar ATPase is a host enzyme necessary for acidification of the endosome and essential for DENV infection. Ideally any negative control(s) used during assay development provide an adequate estimation of the variability of the diverse genomic population, although during assay development this is unlikely to be known. Qiagen included the GFP siRNAs as a negative control on every microtiter assay plate and we added the AllStars Negative Control siRNA during the screen. Post-screen analysis determines that the combined distribution for the negative controls mirrors the genomic distribution, but each negative control behaves differently from the other. The ideal positive control would be an endogenous gene which, when targeted by siRNA, results in a highly reproducible, strong change in the phenotype, relative to the negative control. For assay development, the Qiagen siRNA duplex targeting green fluorescent protein served as a negative control while Qiagen siRNA duplex targeting the endogenous gene ATP6V0C a subunit of the vacuolar ATPase, was used as a positive control.

11. Make the transfection mixture by mixing 360 μ L of RNAimax with 71.6mL of Opti-MEM in the 125mL storage bottles. Mix well but do not create bubbles (*see* Note 8).
12. Run the 70% ethanol partially through the Matrix cartridge thereby creating an air gap between the ethanol and the end of the Matrix cartridge line. Submerge the Matrix cartridge end in PBS and run 15mL of PBS through the line to rinse out the ethanol. Run the PBS partially out of the line creating a new air gap.
13. Submerge the end of the cartridge into the transfection mixture and minimally prime the line.
14. Set the Matrix WellMate to dispense 10 μ L per well for all columns.
15. Load one assay plate. Be sure “A1” is in the upper left. Remove the lid. Press start (*see* Note 9).
16. Replace lid on the first plate and set aside. Fill the remaining plates.
17. Centrifuge the microtiter assay plates at 800 x g for 10–20 seconds to ensure that the transfection mixture reaches the siRNA solution (*see* Note 10).
18. Program termination:
 1. Run the remaining transfection mixture out.
 2. Run 15mL PBS through the cartridge.
 3. Run 15mL 70% ethanol through the line.
 4. . Let the cartridge line sit submerged in 70% ethanol while you prepare the HuH-7 cells for dispensing.
19. Add 3.75mL 1M HEPES to 375mL of DMEM, 5% FBS, PS to create the complete screening medium.
20. Retrieve 3 out of the 5 assigned T-75 flasks containing that day’s HuH-7 cells (*see* Note 11).
21. Remove the growth media from the flasks and rinse the HuH-7 cells with 4mL PBS. Remove the PBS. Then rinse the HuH-7 cells with 1.5 mL of Trypsin/EDTA, 0.25%. Remove the Trypsin/EDTA, 0.25% and discard.
22. Add 1.5mL fresh Trypsin/EDTA, 0.25% to each flask and return to the 37°C incubator for 5 minutes.
23. Remove the Trypsin-treated flasks and tap the flasks in order to dislodge the cells.

⁸53.76mL of Opti-MEM and transfection reagent are needed to fill 14 microtiter assay plates. An additional 7.5mL is needed to prime the Matrix cartridge line, 7.5mL is needed to ensure that line stays filled until the end, and 7.0mL is necessary to ensure that the end of the Matrix cartridge line remains submerged throughout the dispensing step. Importantly, the exact conditions we describe may not result in the best transfections for every cell type.

⁹The Matrix WellMate will move the microtiter assay plate across the deck. Ensure that the vacuum hose does not get tangled with moving parts as it could disrupt delivery of the transfection mixture. Also, it is important to observe the microtiter assay plate orientation because the operator can identify if row or column errors may be introduced due to an unexpected instrument malfunction.

¹⁰According to the manufactures recommendation, the siRNA and transfection mixture should be allowed to incubate for at least 20 minutes. Generally, the trypsin, cell counting and dilution of the HuH-7 cells can be completed easily within this time limit.

24. Add 10mL of the complete screening medium to each flask. Triturate vigorously several times to dissociate the HuH-7 cells from each other. Pool the cell suspension from all three flasks and remove 0.5mL of cell suspension for counting on a hemocytometer.
25. Calculate how much cell suspension is necessary to complete dispensing (*see* Note 12).
26. Prepare the solution in the sterile 500mL storage bottles.
27. Run the ethanol out of the cartridge and rinse with 15mL PBS.
28. Prime the cartridge with 25mL of cell suspension.
29. Check that the dispense volume is set to 50µL per well.
30. Place the first microtiter assay plate into the loading tray with A1 in the upper left.
31. Remove lid and press start. After dispensing the cell suspension, replace lid on the microtiter assay plate and set aside. Continue filling all 14 plates in this manner (*see* Note 9).
32. Set microtiter assay plates on the stainless steel floor of the laminar flow hood and incubate five minutes at room temperature prior to placing all assay plates on a single shelf of the 37°C tissue culture incubators.
33. Time completed _____
34. Program termination:
 1. Run the cell suspension out of the line.
 2. Run 15mL PBS through the cartridge.
 3. Run 15mL 70% ethanol through the cartridge.
 4. Let the cartridge line sit submerged in 70% ethanol for 10min. Then run the ethanol out from the line.

3.3. DENV infection, Day 4

1. Addition of DENV fifty-two hours after siRNA treatment using the Bravo Liquid Handling Platform from Velocity11 for the delivery of 20µL of viral-containing complete screening medium into the microtiter assay plates (*see* Note 6 and Note 13).

¹²For one batch prepare 330mL of a 2.4×10^4 cells/mL solution in a 500mL tissue culture grade disposable bottle. Each well within the microtiter assay plate will receive 0.050mL cell suspension with 1,200 HuH-7 cells per well.

¹³The duration of and calculated multiplicity of infection (MOI) for the initial infection of DENV was optimized to provide consistent and maximal differentiation between the negative and positive control populations. The calculated MOI uses the estimated number of cells in a well and the Vero-derived viral titer (*see* Note 14). The reverse transfection conditions, cell plating density and RNAi duration have been established [9,8,3]. DENV viral production from HuH-7 cells can be observed 20 hours post-infection (data not shown). In order for the genomic screen to identify gene products necessary for any step(s) in the viral life cycle, we permitted DENV to replicate for 42 hours. The Z' factor of 0.87 was observed when an MOI of 0.2 was applied to the cells suggesting an "excellent" assay [13]. All further assay development and genomic screening used this set of conditions.

2. The Velocity11 and Bravo unit are housed within a Baker BioPROtect II biological safety cabinet approved for biosafety level 2 projects.
3. Warm at least 200mL of complete screening media at 37°C.
4. Set an Omni Tray onto position 1 on Bravo deck then fill with 50mL 70% ethanol.
5. Set an Omni Tray onto position 2 on Bravo deck and fill with 50mL 100% ethanol.
6. Set an Omni Tray with fresh Kimwipes onto position 3 on Bravo deck.
7. Set an Omni Tray onto position 4 on Bravo deck then fill with 50mL sterile dH₂O.
8. Set a box of sterile ST70 tips onto position 5 on Bravo deck (*see* Note 18).
9. Set an empty Omni Tray onto position 7 on Bravo deck.
10. Don all appropriate biohazard clothing. The appropriate personal protection equipment will be determined by each institution and may include biosafety level 2 protections including a gown, double layer non-latex gloves and eye protection.
11. Make complete screening medium by addition of 1.25mL 1M HEPES to 123.8mL of the DMEM, 5% FBS, PS into a sterile 125mL bottle.
12. Defrost 4mL of the DENV at 37°C (*see* Note 14).
13. Mix the DENV viral stock gently with a 5mL pipette.
14. Add 3.4 mL of DENV stock to the 125mL of complete screening medium.
15. Mix the diluted viral stock gently 3x with a 25mL pipette.
16. Add at least 50mL of diluted DENV stock into the empty Omni Tray at position 7 on the Bravo deck. Replenish the viral containing media in the Momentary as needed throughout the procedure.
17. Load viral delivery program. (*see* Note 15)
18. Set assay for 14 replications.
19. Obtain microtiter assay plates from the 37°C incubator one plate at a time.
20. Take 1st uninfected microtiter assay plate from the 37°C incubator and transfer it to the Baker BioPROtect II biosafety cabinet and place it on position 8 on the Bravo deck (*see* Note 16).
21. Remove all lids.

¹⁸Remove the ST70 tips from column 1. Wells in column 1 from the microtiter assay plate will not received virus. The uninfected wells are useful for setting background corrections when using automated image analysis which was not discussed by this protocol.

¹⁵The Velocity11 programming software BenchWorks v3.0.0 permits the user to assign predesigned tasks to each position on the deck. The user adjusts the height, volume, speed, and order of the tasks within the software controlling the Bravo unit. The development of the software program is completed well before the screen and must be carefully evaluated for errors by individuals trained to operate the system. At this time, Agilent has discontinued BenchWorks v3.0.0 and replaced it with VWorks.

¹⁶We programmed the Velocity11 software BenchWorks v3.0.0 to dispense 20 µL of virus containing media to each well of the assay plate and mix twice slowly in a volume of 40µL. Finally the tips are touched lightly to the inside of each well to remove any liquid before re-lidding and moving the next assay plate onto position 8 for infection. The assay plates are infected in a predetermined order so that systematic errors may be easier to identify during assay analysis.

22. Run viral delivery program. (*see* Note 16)
23. At the “pause” replace lid on the infectious assay plate on position 8. It is important to note that the plate is now infectious and any spills should be treated using the appropriate biosafety cleanup protocol.
24. Remove the plate from position 8 in the Baker BioPROtect II biosafety cabinet and return to the 37°C incubator. Care must be taken to follow all established biosafety protocols. Return to Step 20. Repeat for each assay plate.
25. Replenish the viral containing media as needed in the Omni Tray at position 7 by addition of 50mL.
26. ST70 tips at position 5 on Bravo deck are reused throughout the screen, The tips are decontaminated inside the Baker BioPROtect II. The viral delivery program ends by washing the ST70 tips in dH₂O, then in 70% ethanol and then in 100% ethanol and finally blotting dry on sterile kimwipes. Tips are left at position 5 on Bravo deck for use in subsequent days.
27. When done, discard all viral containing or exposed liquids into bleach with a final concentration of bleach not to be less than 10%. Each virus-contaminated Omni Tray is exposed to 10% bleach and then discarded into biohazard bags.

3.4. Fixation of HuH-7 cells 42 hours after infection with DENV, Day 6

1. Make 300 mL of PBS with 0.5% Triton-X100
2. Make sure you have on hand ~4500mL PBS-Tween
3. Obtain 300mL ice cold 4% PFA
4. Empty the collection bottles associated with the Biotek ELx405 plate washer (*see* Note 6)
5. Fill the dispensing bottles associated with the Biotek ELx405 plate washer with PBS-tween
6. Prime the Biotek ELx405 plate washer by running 100mL of PBS-tween wash buffer through the system to remove previously used buffer and air bubbles before washing the first plate each day.
7. Add 1.65mL normal donkey serum to each of three aliquots of 165mL PBS-Tween. Label the bottles as indicated and store on ice or at 4°C
 1. Blocking buffer; 2. Primary Antibody; 3. Secondary Antibody and Hoechst
8. Don all appropriate biohazard clothing. The appropriate personal protection equipment will be determined by each institution and may include biosafety level 2 protections including a gown, double layer non-latex gloves and eye protection.
9. Install the VP179 aspiration manifold in the laminar flow hood (*see* Note 17).
10. Remove seven microtiter assay plates from the 37°C incubator.

11. Remove all except 15 μ L of virus containing media from each well using the VP179 aspiration manifold in the laminar flow hood (*see* Note 17).
12. Add 50 μ L of ice cold 4% PFA to each well using the Matrix electronic pipette.
13. Incubate each microtiter assay plate for 12 minutes.
14. After fixation, using the Biotek ELx405, wash each microtiter assay plate. The Biotek ELx405 is programmed to add and remove 50 μ L of PBS-tween to each well 3 times.
15. Permeabilize the cells by adding 50 μ L of PBS with 0.5% Triton X-100 to each well using the Matrix Impact2 Multichannel Electronic Pipette.
16. Incubate each plate for 15 minutes.
17. After permeabilization, repeat wash step 14.
18. Add 30 μ L of the blocking buffer containing PBS-tween and 1% normal donkey serum using the Matrix Impact2 Multichannel Electronic Pipette to each well. Let this first set of plates incubate in this blocking buffer while you treat the second set of seven plates.
19. Return to step 9, and repeat steps 9 through 18 with the second set of seven microtiter assay plates.
 1. The catch-up point is step 18.
 2. After addition of the blocking solution to the first plate of the second set, start timer to incubate for 50min
20. During the incubation period, add 165 μ L of 4G2 antibody to the 165ml of PBS-tween, 1% normal donkey serum labeled “primary antibody” bottle prepared earlier.
21. After 50min, wash each microtiter assay plate using the Biotek ELx405 as in step 14.
22. Add 30 μ L of “primary antibody” solution to each well. Start timer to incubate 1h right after addition to the first microtiter assay plate.
23. During the incubation period, add 82.5 μ L of Alexa488 and 165 μ L Hoechst to PBS-tween, 1% normal donkey serum labeled “Secondary Antibody and Hoechst” bottle prepared earlier.
 1. Work with least ambient light possible to protect the fluorescent dyes on Alexa488.
24. After 1 hour, wash each microtiter assay plate using the Biotek ELx405 as in step 14.

¹⁷The VP179 aspiration manifold is a simple lever system that consistently removes a predetermined volume of virus containing media from all 384 wells of a single assay plate. This system is used to remove all except 15 μ L of media to ensure that cells do not dry out before adding 4% PFA.

25. Add 30 μ L of “Secondary Antibody and Hoechst” mixture to each well. Start timer to incubate 1h right after the first plate is done. Incubate under a piece of aluminum foil to protect the plates from light.
26. After 1 hour, wash each microtiter assay plate using the Biotek ELx405 as in step 14.
27. Add 50 μ L PBS.
28. Hermetically seal, clean imaging surface with 95% ethanol, and set up the Array Scan.

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