Chapter 10

Methodology for Identifying Host Factors Involved in West Nile Virus Infection

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Abstract

The West Nile virus (WNV) infection is a major medical problem for humans and some domesticated animals. WNV infection of host cells involves the interplay of the virus with several host factors. Identification of the host factors impacting on WNV infection can enhance our understanding of virus infection mechanisms, host immune defense mechanisms, and also reveal novel host targets that can be developed as antivirals. RNA interference (RNAi) is a highly efficient genetic tool to discover host genes involved in WNV infection at a genome scale. Here, we describe a protocol for conducting human genome wide RNAi screen to discover novel host factors associated with WNV infection of human cells.

Key words West Nile virus, WNV, RNA interference, RNAi, siRNA, Transfection, High-content microscopy

1 Introduction

WNV is a single plus-stranded RNA genome containing flavivirus [1]. Infection by WNV has been a recurrent human health problem in different parts of the world [2–4]. Despite the health threat posed by WNV, no effective therapeutics or vaccine is available against human infections caused by WNV [5].

Understanding the molecular mechanisms defining WNV–host cell interactions is essential for discovering novel therapeutic targets and approaches against this virus. WNV genome encodes for three structural and seven nonstructural proteins. Given their obligate intracellular life cycle with a minimal genome, it is apparent that the WNV will have to exploit a diverse compendium of host cellular molecules for completing their replication. Such host factors that support WNV infection is termed host susceptibility factors (HSFs). Contrary to this, the host cells would have evolved molecules to resist WNV infection (termed host restriction factors, HRFs). Therefore, to understand the molecular aspects of virus–host cell interactions, it is essential to discover the host factors

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involved in both supporting and restricting WNV infection. The dependence of viruses on host factors during infection also catalyzed the formation of the novel concept of host-targeted antiviral discovery, in which it is envisaged that essential infection supporting host molecules can form therapeutic targets against viruses [6].

A variety of approaches were used previously to identify the host molecules associated with WNV infection. Some of these approaches included gene expression profiling using transcript measurements, and protein–protein interaction screens using WNV proteins as baits [7–9]. Recently, a powerful forward genetics approach, RNA interference, has been increasingly exploited to dissect virus–host interactions [10, 11]. The RNAi provides unprecedented ability to discover host genes involved in supporting and restricting viral infections. Two previous genome wide RNA interference screens performed respectively in drosophila and human cells identified several host genes impacting on WNV infection [12, 13].

Here we provide a detailed account of the methodology for conducting large-scale high-throughput format RNAi screening to discover novel host genes regulating WNV infection of human cells. A schematic of the RNAi screening is given in Fig. 1.

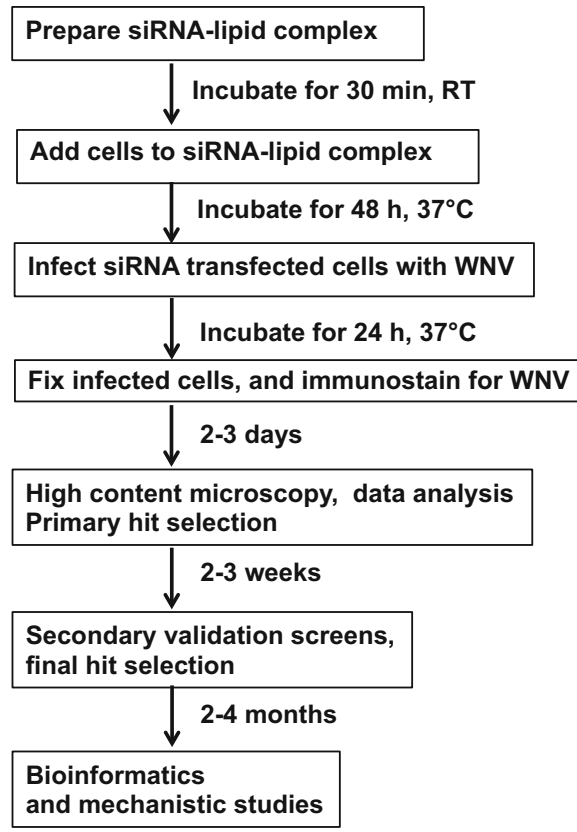

Fig. 1 Schematic of RNAi screening to identify human host factors involved in WNV infection. *h* hours, *RT* room temperature, *siRNA* small interfering RNA

2 Materials

2.1 Cell Culture

1. HeLa cells (ATCC catalogue number CCL-2).
2. Dulbeccos Modified Eagle Medium (DMEM, high glucose), containing 20 % fetal bovine serum (heat inactivated) as well as serum free.
3. 0.25 % trypsin-EDTA.
4. Cell culture incubator, 5 % CO_2, 37 °C.
5. Pipette, cell culture type, 5, 10, and 25 ml.
6. Laboratory Pipettes, various sizes.

2.2 siRNA and Transfection Reagents

1. Positive control siRNA duplexes that blocks WNV infection, targeting human vATPase subunits ATP6V0A1 (gene ID 535) and ATP6V1A (gene ID 523). Dharmacon catalogue numbers for the siRNAs targeting ATP6V0A1 and ATP6V1A respectively are M-017618-00 and M-017590-00. Prepare a 1 μM stock of the siRNA in the resuspension buffer, and store at –20 °C.
2. Negative control siRNA that does not affect WNV infection (e.g., Dharmacon catalogue number D-001206-13-05). Prepare a 1 μM stock of the siRNA in siRNA resuspension buffer and store at –20 °C.
3. siRNA libraries. Human genome wide siRNA library containing a pool of four unique siRNAs targeting each human gene (Dharmacon catalogue number G-005000-05), arrayed at 1 μM concentration in siRNA resuspension buffer. The siRNA library can be stored at –20 °C. The human genome wide siRNA library is plated into 384 well master plates in all wells except column 1, 2, 23, and 24. The columns 1, 2, 23, and 24 contains only siRNA resuspension buffer.
4. Transfection reagent Dharmafect 1 (Dharmacon catalogue number T-2001-01).
5. siRNA resuspension buffer (Dharmacon catalogue number B-002000-UB-100).
6. Multichannel (12-channel) digital pipettes (Eppendorf ResearchPro 300 μl, and 100 μl) or automated liquid handler with 384-well head and sterile disposable tips (i-Pipette Pro, Apricot Designs).
7. Multichannel reagent reservoir compatible for 12-channel (ThermoFisher catalogue number 15075) or 384-channel (Thermo Scientific catalogue number 1064-05-7).
8. Imaging quality black wall clear bottom sterile cell culture treated 384 well plates (Corning catalogue number 3712).
9. Adhesive seal (e.g., VWR catalogue number 60941-124) for covering 384-well plates.
10. 50 ml conical polypropylene tubes.

2.3 Viral Infection

1. West Nile virus stock (10^6 pfu/ml).
2. Biosafety Level-3 laboratory.
3. 10% Bleach.

2.4 Immunostaining

1. Paraformaldehyde (4%) solution in phosphate buffered saline.
2. Anti-West Nile Virus/Kunjin Antibody detecting Envelope protein (Millipore catalogue number MAB8151).
3. Blocking buffer (phosphate buffered saline, 0.05% Tween-20).
4. Permeabilization buffer (phosphate buffered saline, 0.1% Triton X-100, 0.05% Tween-20).
5. 3 μg/ml DAPI (4′,6-diamidino-2-phenylindole, sigma catalogue number D-1388) in PBS.
6. Alexa Fluor 488 conjugated anti-mouse secondary antibody (1:2000-fold dilution of a 2 mg/ml antibody stock).

2.5 High-Content Fluorescence Microscopy

1. ImageXpress Micro high-content microscope (Molecular Devices Corporation).

2.6 Data Generation and Analysis

1. MetaXpress software.
2. Microsoft excel.

3 Methods

The methods described here explain how to perform RNAi screen in 384 well plate format to identify host factors important for WNV infection of human derived HeLa cells. This procedure employs the detection of WNV in cells through fluorescent dye Alexa Fluor 488 conjugated secondary antibody based immunostaining, and visualization by fluorescence microscopy. The siRNA transfection protocol described here involves simultaneous cell plating and siRNA transfection. In this procedure, each gene will be targeted by a pool of four unique siRNAs. The final concentration of each one of the individual siRNAs transfected into the cells is 12.25 nM (this equals to 50 nM for the combined four siRNAs in each pool). The methods given below describe how to perform the screen using both manual multichannel (12-channel) electronic pipette and automated 384-channel liquid handler. The steps below are described for performing RNAi screen in a single 384-well plate. Users can scale-up this protocol depending on the number of plates involved in specific screening experiments.

3.1 Preparation of siRNA Screen Plate

All procedures should be carried out within a biosafety cabinet.

1. Prepare a map for the 384-well plates intended for screening (termed Screen Plate), marking the location of control and test siRNA identities. Columns A3 to A22 contains siRNA library. Wells B2, C2, and D2 will receive negative control siRNA. Wells E2, F2, and G2 will receive positive control siRNA. All other wells are either left as additional control wells that would not receive siRNA, or could be used for plating additioal negative and positive control siRNAs.
2. Thaw the 1 μM siRNA master stocks at the room temperature for 30 min. Spin the plates for 2 min at 200 × *g*, to settle any liquid that may be present on the walls of the wells. Remove the plate seals.
3. Fill a reagent reservoir with 350 μl of siRNA resuspension buffer. Transfer 8 μl of siRNA resuspension buffer into all wells of the Screen Plate, using 12-channel electronic pipette. The liquid should be dropped onto the side of the well close to the bottom of the plate.
4. 2. From the 1 μM master stocks of the human genome wide siRNA library (arrayed in the 384-well plate), transfer 2 μl of siRNA into the Screen Plate, using either automated liquid handler or 12-channel electronic pipette. The siRNA should be transferred to the Screen Plate in the exact same order as in the master plate.
5. Remove 2 μl of the siRNA resuspension buffer from the wells B2-G2 (wells designated to receive negative and positive siRNA controls) using a 10 μl pipette. Then, add 2 μl of the negative controls siRNA stocks (stock of 1 μM) into the wells B2-D2, and positive control siRNAs into the wells E2-G2.
6. Gently tap the plate using hand, and let the plate sit at room temperature for 10 min, to disperse the siRNA.

3.2 Preparation of siRNA Transfection Complexes

(*Note*: go to Subheading 3.2 only after completing Subheading 3.1.)

The amount of Dharmafect1 transfection reagent needed for transfection per well is 0.05 μl. All steps should be carried out in a laminar flow cell culture hood (*See* **Notes 1–9**).

When using a manual multichannel (12-channel) electronic pipette:

1. Add 24.2 μl of Dharmafect1 into 4.84 ml of serum-free DMEM in a 50 ml conical tube, vortex at medium speed for 30–60 s. (In order to ensure efficient pipetting, an additional volume of transfection mix needed for 100 wells in excess is required. Therefore, while preparing transfection reagent for a single 384-well plate, the actual generated reagents will be for 484 wells).

2. Transfer the DMEM containing Dharmafect1 to a fresh sterile reagent reservoir using 10 ml pipette.
3. Aspirate 300 μl of the HeLa cell containing DMEM using a 300 μl manual multichannel (12-channel) electronic pipette, and drop 10 μl into each well of the Screen Plate, 15 times. Continue this process until all wells are supplied with the transfection complex.
4. Incubate the Screen Plate for 30-40 min at the room temperature for siRNA-lipid transfection complex formation.

When using automated 384-channel liquid handler Apricot I-pipettor with 125 μl capacity tips:

1. Add 57.6 μl of Dharmafect1 into 11.52 ml of serum-free DMEM in a 50 ml conical tube, vortex at medium speed for 30 s. This step prepared sufficient reagent for transfecting into three 384-well plates, although we are actually transfecting into only one plate. The 384-well block size reagent reservoir used for automatic liquid handler requires around 10–12 ml liquid to fully cover its bottom. Therefore, in order to ensure efficient pipetting, at least 10–12 ml [enough for transfcetion into three 384-well plates] of transfection complex should be added to the reagent reservoir.
2. Transfer the DMEM containing Dharmafect1 to a reagent reservoir using 10 ml pipette.
3. Aspirate 15 μl of the DMEM containing Dharmafect1 using automated 384-channel liquid handler, and drop 10 μl into the Screen Plate. Discard the remaining 5 μl of the DMEM containing Dharmafect1 from the tips.
4. Incubate the Screen Plate for 30 min at the room temperature for siRNA-lipid transfection complex formation.

3.3 Preparation of HeLa Cells for Transfection

All steps should be carried out in a laminar flow cell culture hood.

This step should be started immediately after performing Subheading 3.2, and performed in such a way that the cells should be ready when the 30 min incubation period required for siRNA-lipid transfection complex formation is completed.

1. Remove two 10 mm culture dishes containing 70–80% confluent HeLa cells from 37 °C incubator into biosafety cabinet, aspirate the medium, rinse cells gently with room temperature PBS twice, and add 1.5 ml of 0.25% Trypsin-EDTA.
2. Incubate the trypsin treated cells in 37 °C incubator for 3–5 min.
3. Transfer the dish containing trypsin treated cells to the biosafety cabinet, add 7 ml of 10% serum containing DMEM to the cells, and dislodge adherent cells by passing the medium gently

through the pipette multiple times. Spin the cells in a 50 ml tube for 5 min at 200 × *g*. Resuspend the cell pellet in fresh DMEM containing 20% FBS, and count the cells using hemacytometer. Prepare a diluted stock of HeLa cells at a concentration of 100,000 cells/ml in DMEM containing 20% FBS.

3.4 Transfection of siRNA into HeLa Cells

When using a manual multichannel (12-channel) electronic pipette:

1. Transfer 9.68 ml of diluted HeLa cell (100,000 cells/ml in DMEM containing 20% FBS) stock into a fresh sterile reagent reservoir (ThermoFisher catalogue number 15075).
2. Aspirate 300 μl of the HeLa cell containing DMEM using a 300 μl manual multichannel (12-channel) electronic pipette, and drop 20 μl into each well of the Screen Plate, 15 times. Continue this process until all wells are supplied with the cells.
3. Incubate the Screen Plate for 48 h in a cell culture incubator, 5% CO_2, 37 °C.

When using automated 384-channel liquid handler Apricot I-pipettor with 125 μl capacity tips:

1. Transfer 12 ml of diluted HeLa cell (100,000 cells/ml in DMEM containing 20% FBS) stock into a fresh sterile reagent reservoir (Thermo Scientific catalogue number 1064-05-7).
2. Aspirate 25 μl of the HeLa cell containing DMEM using 125 μl capacity tips, and drop 20 μl into each well of the Screen Plate, six times. Continue this process until all wells are supplied with the cells.
3. Incubate the Screen Plate for 48 h in a cell culture incubator, 5% CO_2, 37 °C.

3.5 Infection of siRNA Transfected HeLa Cells in 384-Well Plates

The virus will be added into each well in a volume of 10 μl. Dilute the stock virus in serum-free DMEM in such a way that 10 μl of the diluent medium contains enough virus particles to give an MOI of 0.3 (*See* **Note 10**).

When using a manual multichannel (12-channel) electronic pipette:

1. Transfer diluted virus stock into a fresh sterile reagent reservoir (ThermoFisher catalogue number 15075).
2. Aspirate 300 μl of the virus stock using a 300 μl capacity manual multichannel (12-channel) electronic pipette, and drop 10 μl into each well of the Screen Plate, 15 times. Continue this process until all wells are supplied with the virus.
3. Incubate the Screen Plate for 24 h in a cell culture incubator, 5% CO2, 37 °C.

When using automated 384-channel liquid handler Apricot I-pipettor with 125 μl capacity tips:

1. Transfer diluted virus stock into a fresh sterile reagent reservoir (Thermo Scientific catalogue number 1064-05-7).
2. Aspirate 15 μl of the virus stock using 125 μl capacity tips, and drop 10 μl into each well of the Screen Plate, 15 times. Continue this process until all wells are supplied with the virus.
3. Incubate the Screen Plate for 24 h in a cell culture incubator, 5 % CO_2, 37 °C.

3.6 Fixing and Processing of siRNA Transfected HeLa Cells in 384-Well Plates for IFA

1. Arrange 16% PFA solution, and 10% bleach in the culture hood. Transfer the plates into culture hood.
2. Using pipette, aspirate 30 μl from each well, and discard into 10% bleach tray. This will leave each well with 20 μl of culture medium.
3. Dispense 60 μl of 16% PFA into each well, using either manual electronic pipette or automated 384-channel liquid handler Apricot I-pipettor. Spray 16% PFA to the entire plate to inactivate any medium containing virus present outside the wells.
4. Incubate in the safety cabinet for 20–30 min at the room temperature.
5. Aspirate 80 μl of the liquid from each well.
6. Dispense 80 μl PBS to each well, and incubate for 5 min. Spray PBS to the entire plate to wash any PFA present outside the wells.
7. Aspirate 80 μl of the liquid from each well.
8. Dispense 80 μl PBS to each well, and incubate for 5 min.
9. Repeat **steps 7** and **8** four times, and remove as much of the PBS from the well as possible.
10. Add 30 μl permeabilization buffer, and incubate for 30 min.
11. Remove permeabilization buffer by aspirating 30 μl of the liquid from each well, dispense 80 μl PBS to each well, and incubate for 5 min.
12. Repeat **steps 7–8** four times.
13. Add 30 μl block buffer, and incubate for 30 min.
14. Remove block buffer by aspirating 30 μl of the liquid from each well.
15. Add 20 μl primary antibody (1:500 diluted in block buffer), and incubate overnight at 4 °C. Keep the plate wrapped in aluminum foil or protected from light.
16. Remove primary antibody by aspirating 20 μl of the liquid from each well, dispense 80 μl PBS to each well, and incubate for 5 min. Repeat **steps 7–8** four times. Keep the plate wrapped in aluminum foil or protected from light.

17. Add 20 μl anti-mouse-Alexa488 secondary antibody (1 μg/ml in PBS), and incubate for 2 h at 37 °C. Keep the plate wrapped in aluminum foil or protected from light.
18. Remove secondary antibody by aspirating 20 μl of the liquid from each well, dispense 80 μl PBS to each well, and incubate for 5 min. Repeat **steps 7–8** four times. Keep the plate wrapped in aluminum foil or protected from light.
19. Add 20 μl DAPI solution (1:2000 diluted in block buffer), and incubate for 30 min at room temperature. Keep the plate wrapped in aluminum foil or protected from light.
20. Remove DAPI solution by aspirating 20 μl of the liquid from each well, dispense 80 μl PBS to each well, and incubate for 5 min. Repeat **steps 7–8** two times. Keep the plate wrapped in aluminum foil or protected from light.
21. Seal the plate with adhesive seal. Keep the plate wrapped in aluminum foil or protected from light.

3.7 High-Content Imaging of WNV Infected HeLa Cells in 384-Well Plates

This description is based on the high-content fluorescence microscope ImageXpress Micro by Molecular Devices. Users may use different makes of high-content fluorescence microscope (*See* **Notes 11–19**).

1. Turn the microscope on.
2. Load acquisition software.
3. Set imaging parameters for imaging at 4× magnification. Green channel for virus, and blue channel for cell number.
4. Open microscope loading chamber lid, load the 384-well plate with A1 position aligning with the marked position on the plate holder, and close the lid.
5. Adjust the focus by selecting A1, A12, and A24 wells.
6. Give the command for acquisition.
7. When acquisition is completed, remove the plate from the microscope, and turn off the microscope.

3.8 Infection Quantification

1. Following the instructions provided in the MetaXpress software user manual, select the "multiwavelength cell scoring" algorithm and set the analysis parameters using the newly acquired images of WNV infected cells.
2. The selected parameters should detect both DAPI stained nucleus, Alexa-488 stained WNV.
3. The quantified outcomes from the analysis by the software should generate information on "total number of cells, total number of WNV positive cells, and percentage of WNV positive cells" in a linked excel spreadsheet.

4. Run the software to quantify WNV infection.

3.9 Data Analysis Using Mean Based Z-Score Determination

After the completion of the automated image analysis, transform the percent infection data generated in the excel spreadsheet, as given below.

1. Determine the mean percent infection of the entire plate in the excel spreadsheet using its mathematical processing options.
2. Next, calculate the standard deviation of the mean percent infection of the entire plate in the excel spreadsheet using its mathematical processing options.
3. Determine the *Z*-score using the formula $z = x - \mu/\sigma$ (x denotes the percent infection in each well, σ denotes the standard deviation of the mean percent infection of the entire population, μ denotes the plate mean percent infection).
4. Sort the phenotypes obtained for each well based on the *Z*-score corresponding to the wells, from low to high.
5. Define a *Z*-score cut off value for hit selection.

4 Notes

The following points may help to further enhance the success of the RNAi screening assays.

1. It is desirable to exclude one or two outer layers of wells to reduce the chances of edge effect occurring.
2. Some types of cells that are less adherent or nonadherent can be made adherent using specially coated plates (e.g., CellBIND plates, Corning, Cat# 3683) which received treatments that enhances the ability of the dishes to anchor the cells. It was observed that plating siRNA into these plates first (e.g., for forming the complex with lipid) often leads to a reduction of knockdown efficiency by more than 70%. While the exact reason is unknown, one possibility is that the extra treatment applied to these dishes may prevent the siRNA complex from entering the cells optimally.
3. If the liquid or cells are stuck to the walls of 384-well plate while dispensing, centrifuge the plates at around $200 \times g$ for 20–40 s.
4. To avoid air bubble created in the wells of the 384-well plate while dispensing liquids, always pipette at least 3–5 μl more liquid than is actually required.
5. To resuspend siRNA, one can also use alternative buffers than the one described here, or even RNase-free water.
6. Use of manual multichannel pipette that are not electronically controlled can introduce significant amount of variability, and can essentially compromise the quality of the assay.

7. Use of desiccated chambers to thaw siRNA plates will reduce water condensation around the outer regions of the plates during thawing.
8. For transfecting siRNA into faster growing cells, reduced initial plating cell number or growing in less serum containing medium may help to slow down cell reaching confluence.
9. The amount and type of transfection reagent useful for various cell types can vary greatly. Therefore, optimization experiments using various doses of different transfection reagents are necessary.
10. Adding virus in a very small volume (e.g., 1 or 2 μl) of inoculum into the wells can occasionally lead to localized infection (on the cells only around the area where the virus was dropped) than a uniform distribution of infected cells across a well. Infection with 10–20 μl of inoculum often yields the best result.
11. During automated scoring, the threshold intensity of immunostaining selected for scoring positive and negative signals can greatly influence the nature of the results. For example, if a 24 h infection results in 20% and 10% of the cells having very strong and weak signals for WNV respectively, then excluding the weak signal giving cells from automated scoring may enable accurate identification of infection enhancing knockdowns. This is because, under infection enhancing conditions, the weak signal giving cells will mostly also become strong signal emitting cells and hence will be readily picked by the software.
12. There are many different of statistical approaches to analyze the RNAi screen raw data and select the potential hits. Some of these are explained in several previous reports [14].
13. Many times the fluorescent tag (e.g., alexa-488) may detach from the conjugated antibody and stay in the cells. This can seriously interfere with the automated image scoring because the software may interpret the signal from fallen dye as immunostained WNV. It is always desirable to pre-test the secondary antibody to ensure that it does not cause dye detachment-based background signal.
14. Imaging of the immunostained cells can be performed at different magnifications, depending on the type of information sought by the investigator. Imaging at 5× magnification is often enough to identify the presence or absence of infection. However, in general, imaging at 5× will not enable the investigator to clearly understand the differences in localization of fluorescent signals for the WNV within the cells. Imaging at higher magnifications (e.g., 10× or 20×) may provide better information on the subcellular localizations of WNV.

15. There are no standard rules on how many replicates of the primary screen should be conducted. The primary RNAi screen can be performed in multiple ways cost effectively, such as: (a) a single round of full genome screen, followed by selection of the hit genes, and re-testing of all hits only in triplicates; (b) perform whole genome RNAi screen in duplicates; or (c) perform whole genome RNAi screen in triplicates.
16. Validation of the primary RNAi screen hits can be performed in many ways. There are two major goals for the validation of primary RNAi screen results: (a) determine whether a siRNA that gave a phenotype in the primary screen is having on-target specificity. This can be achieved by the use of multiple independent siRNAs tested against the same gene, and complementation using siRNA-resistant version of the same gene, among other possible approaches; and (b) whether the observed phenotype is due to any assay bias. This can be validated by using alternate viral load measurement assays such as plaque assay, PCR based quantification of viral RNA and detection of intracellular viral protein levels.
17. From a typical genome wide RNAi screen for virus–host interactions, assuming that all tested siRNAs are efficiently knocking down their intended target genes, three kinds of phenotypes are possible: (a) a gene knockdown that enhances viral infection. Such genes will be direct host restriction factors or genes that regulate indirect cellular processes negatively impacting on viral infections; (b) a gene knockdown that reduces viral infection. Such genes will be direct host susceptibility factors or genes that regulate indirect cellular processes positively impacting on viral infections; and (c) a gene knockdown that does not alter viral infection. Such genes do not play any direct or indirect role in viral infection.

    However, in reality, the currently available commercial siRNA libraries are not validated for their knockdown efficiency and on-target specificity. Therefore, it is very likely that one might identify several false negative and false positive hits from each RNAi screen. While false positive hits will likely be identified and eliminated through subsequent validation secondary RNAi assays, false negatives are typically never identified (unless independent RNAi screenings using additional libraries or validated siRNAs are performed).
18. Altering the time and type of measurements of viral load can provide different information. The 24 h infection described here mostly will only report changes in viral infection arising from steps preceding virion assembly and secretion.
19. A typical human whole genome RNAi screen involves targeting approximately 18,000 genes. This many genes will often require around fifty-sixty 384-well plates depending on the arraying of the siRNAs.

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