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A Genome-wide RNAi screening method to discover novel genes involved in virus infection

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Abstract

Systematic and comprehensive analysis of host cell proteins involved in virus infection has been difficult in large part due to the lack of robust unbiased methods for their identification. Recent technological breakthroughs allowing development of cell-based genetic screens have greatly facilitated our understanding of virus-host interactions. These include instrumentation for processing in microtiter plates (e.g. 384 well), coupled with sensitive readers and off-the-shelf analysis and informatics pipelines. Because viruses are a significant threat to human health, a better understanding of the cellular factors that impact infection would pave the way for the development of new therapeutics. Here we describe the development and implementation of a genome-wide siRNA screen against a virus using human cells.

1. Introduction

Viruses are obligate intracellular pathogens with a limited coding capacity and thus require host cell factors for their replication. Viral proteins interact with and regulate their host cell environment to facilitate replication using sophisticated strategies that interface with normal biological processes. Viruses have also developed strategies to subvert host restriction mechanisms. Understanding how viruses exploit cellular machinery for their own replication and propagation has greatly contributed to our knowledge of fundamental principles of cell biology. Considerable attention has been devoted towards development of systems to comprehensively analyze virus-host interactions including genome-scale RNAi screens. In recent years a number of studies have reported genome-wide RNAi screen for viruses identifying hundreds of novel players in the virus-host interface (3-17). Additionally, other unbiased technologies including the proteomics (yeast two hybrid, shotgun proteomics) and transcriptomics (microarrays, RNA-seq) can be coupled with RNAi screening to allow for the rapid and systematic discovery of cellular genes that impact viral infection. Although the

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functions of many of the genes identified in genome-wide RNAi screens remains to be defined, these studies have greatly facilitated our knowledge of the interface between these viruses and their host, and will undoubtedly lead to continued insight into the cell biological roles of many of these genes.

There are two basic genetic screening approaches that can be applied to study virus-host interactions. First, gain-of-function strategies can be used to probe gene function by ectopically expressing cDNAs. The recent development of fully sequenced, full-length, arrayed cDNA libraries (e.g. MGC collection) has expanded the utility of these approaches. Second, loss-of-function screens relying on RNAi technology has uncovered many new aspects of the host-pathogen interface. There are a number of RNAi tools that can be used for this: small interfering RNAs (siRNAs), short hairpin RNAs (shRNAs) and long double-stranded RNAs (dsRNAs), which are commercially available, and are the most common reagents. Long dsRNAs are widely used in insect systems providing robust knock down and low off-target effects. Furthermore, microRNA mimic and inhibitor libraries made it possible to perform high throughput screens using these reagents and newly emerging technologies including CRISPR/Cas9 will open up more strategies for screening.

2. A general protocol for siRNA image-based screen in mammalian cells

Based on our previous studies applying RNAi screening against vesicular stomatitis virus (VSV) and Sindbis virus (SINV), in the following section we have established a straightforward pipeline to identify genes that impact virus-host interactions (Fig.1). With minor modifications, this protocol can be applied to other cellular or viral systems to perform siRNA screening.

2.1 Materials required

2.1.1 siRNA reverse transfection—Genome-scale siRNA library (e.g. Ambion Silencer Select, Dharmacon On Target Plus etc.)

Control siRNAs with no known sequence homology with human genes (e.g. Silencer negative control siRNA, Ambion; OnTarget Plus non-targeting siRNA, Dharmacon)

Cell death control siRNA (Allstars HS cell death, Qiagen; Tox control, Dharmacon)

Other positive control siRNAs such as siRNA against RAB5A or ATP6V0B, which are host genes required for virus entry through endosomal uptake

384 well plates (black clear bottom, Corning 3712)

Automated liquid handling device (WellMate, Thermo scientific)

OptiMEM reduced medium (Life Technologies)

Transfection reagents such as RNAiMAX(Life Technologies)

Transfectable adherent cell lines such as HeLa

Filter tips, Multi channel pipettes.

2.1.2 Cell Culture—Tissue culture hood and incubator

Dulbecco Modified Eagle's medium with high glucose (Invitrogen) supplemented with serum and antibiotics

Trypsin-EDTA (0.05%), Invitrogen

2.1.3 Virus infection—Virus expressing a reporter gene such as green fluorescent protein (GFP). In this case we used vesicular stomatitis virus expressing GFP (VSV-GFP). Alternatively, a viral antigen can be monitored using antibodies.

2.1.4 Fixing and staining—4% formaldehyde or 4% paraformaldehyde solution in Phosphate buffered saline (PBS) PBS, Invitrogen

0.1% TritonX-100 in PBS

4,6-Diamidino-2-phenylindole (DAPI) to counter stain the nuclei

Plate washer (Biomek Fx, Beckman Coulter)

Plate sealing adhesive film

2.1.5 Automated imaging and image processing—ImageXpress Micro (Molecular Devices)

Metaxpress software (Molecular Devices)

2.2. Acquiring images using the ImageXpress Micro and image analysis

For imaging, we use the DAPI channel to detect the nuclear stain and the FITC channel to detect viral antigens, in this case GFP for a GFP expressing virus. Next, the imaging protocol is optimized. We capture images at 10X magnification, and obtain 4 sites per well.

We use laser based focusing and adjust the z height to obtain a focused image in each channel and save the settings. If possible, the plates are barcoded, and a robot is used to feed the plates to the imager. Once the images are captured, the image analysis settings are optimized. We use tools available in the software to segment the DAPI channel to identify the nuclei and the GFP channel to identify virally infected cells. In Fig.2 we show the segmentation of the image to allow us to measure cell number and percent infection in a high content assay. Image segmentation to identify DAPI positive cells is performed by defining the minimum diameter of the object and intensity of the nuclear stain (to separate from background). Likewise, the GFP signal is segmented (Fig.2). The software analyzed each site and each well across the plate. Verify that the uninfected wells have no GFP positive cells and that the Cell Death siRNA-treated wells have no cells. Save the protocol.

2.3 Assay optimization

There are a number of important considerations in performing and optimizing cell-based screening and often the assay optimization takes longer than the actual screen. Because these assays require robust differences, easily transfectable cell lines with stable properties (e.g. HeLa, A549, U2OS) are often used for high throughput screens. To optimize transfection efficiency, a range of transfection reagents, dilutions of the transfection reagent, and a range of cell densities are used to obtain the highest level of cell death with the Cell Death siRNA. This is a robust and convenient readout for the efficiency of siRNA transfection. Once the transfection conditions are optimized, the second step is to optimize the virus infection including the MOI and timing. A range of MOI and times of infection should be tested in order to obtain the desired phenotype. Most often the question being asked dictates the MOI and time of infection. For example, if an early stage of virus infection is being investigated, a high MOI and shorter time of infection is used. In contrast a low MOI and longer time of infection is used to investigate multi-cycle infections. To monitor viral infection many investigators have relied on the use of virally-expressed reporters because they are the most robust readouts; however, many viruses cannot be readily reverse engineered necessitating the use of antibodies (e.g., viral antigens) or more indirect read-outs (e.g., cytotoxicity). While the goal is to identify siRNAs that impact viral infection, many siRNAs impact the health of the cells and thus make interpretations challenging. Therefore, quantification of cell number is essential and this is simple using a high content assay. This also allows one to normalize the levels of infection to the numbers of cells in the well. This makes high content screening assay advantageous over plate-based assay where an additional step is required to obtain toxicity information. Statistical tests are used both to optimize the assay (Z' factor >0.5 (which is a measure of assay robustness) (20) and coefficient of variation, $CV < 15\%$ is standard) and to identify 'hits'. A positive control which blocks infection should be used to optimize the screen for genes that promote infection and a second positive control that restricts infection will allow optimization for that class of genes. Fig.3 shows optimization using positive control siRNAs. The negative control siRNA gave 73% infection where as positive control siRNA gave $<1\%$ infection. In this setting, this assay has Z' factor > 0.5 . We found that if you are trying to identify pro-viral host factors, targeting 60-70% infection for the negative control siRNA is optimal while 10-15% is more optimal when screening for restriction factors.

2.4 siRNA library design and plate design

The design of siRNA library plays an important role in how hits are identified and how candidates are validated. The initial human genome-wide screens used pooled siRNAs-containing libraries with 3-4 independent siRNAs targeting each gene. Although this format is cost-effective and increases the throughput, it is more vulnerable to identify false positives. In the pool of 4 siRNAs if one siRNA has the phenotype of interest, then it is identified as a hit. This approach often resulted in low validation during the secondary assay which requires at least two siRNAs to emerge with the same phenotype. Alternatively, some researchers used a 2×2 format where pools of two siRNAs targeting each gene per well were spotted with two wells representing each gene. Therefore, if both wells were identified, then it is likely that there are two independent siRNAs against the same gene with the same phenotype. Recently siRNA libraries are available with three siRNAs targeting each gene

and one siRNA is plated per well. Given the high degree of false positives, secondary screens are used to validate the gene list using any of these libraries. We suggest using siRNAs from an independent manufacturer and of course with different seed regions. At least two independent siRNAs with the same phenotype is commonly used to define a 'validated' gene set. Of course, how the 'hits' are selected is also important. We typically require a Robust Z score of >2 or <-2 in replicate for percent infection and low cytotoxicity ($<30\%$ death) (see 2.8).

There are additional technical issues that can be impacted by the layout of the plates for screening. Since the wells have low volumes there are evaporation issues that are highest in the outer wells leading to potential artifacts. One way to mitigate this would be to leave the outer columns empty, but this is largely impractical. Another important part of the screen is to add positive and negative control siRNAs to each plate. Of course, these are best left off the edges to maximize the likelihood of robust data for the downstream validation of each plate. Figure 4 shows a representative assay plate map. Column 2 and 23 (shaded green) are open for the negative and positive control siRNAs. The library siRNAs-containing wells are shaded in gray.

2.5 Pilot screen

Since each gene is usually only tested in duplicate high accuracy is critical to identify potential candidates. Thus, a pilot screen is performed to test the assay under screening conditions to verify that there is little plate-to-plate and day-to-day variability using all of the equipment that will be used for the genome-scale screen. In the pilot screen 1-2 plates are used in the final screening format to test data distribution and whether statistical tests can identify the positive controls spiked into the plates and that the 'hit' rate is less than 5%. Some researchers use small targeted libraries such as kinases, while others use a plate from the library that has diverse gene types. This pilot screen sets up the standard-operating-procedures (SOPs) that will be used for the full screen. The statistical parameters used in the optimization phase should be calculated to determine if the data is normally distributed and if the parameters are within the ranges seen previously including the Z' factor (see 2.8). A Z' factor of <0.5 for the controls, a non-normal data distribution for the pilot screen or the lack of concordance between the two pilot screens is a serious concern and in such cases it is wise to go back and optimize the screen again.

2.6 Method

2.6.1 siRNA reverse transfection

1. Check the HeLa cells under the microscope to ensure there is no contamination and that there is sufficient cell density to move forward.
2. Pre-warm Optimem, DMEM+10%FBS+Penn/Strp/Kan at 37°C .
3. Use automated liquid handling to array the siRNAs in 384 well format. Generally the siRNAs are pre-plated in 384 well format using robotics and frozen at -80°C . The final concentration of the siRNAs is 20 nM. Defrost assay plates at room temperature (~ 30 min).

4. Centrifuge the assay plates ~500g for ~2-3min to ensure siRNAs are on the bottom of the plate.
5. Assemble the WellMate machine. Prime with 70% ethanol and keep it ready.
6. Array 5 μ l of the 200nM siRNA dilution into appropriate wells of the assay plates. Minimum of 4 wells for each control reagents should be spotted in each plate.
7. Dispense OptiMEM to a 125 ml sterile nalgene bottle
8. Take out the Lipofectamine RNAiMAX from 4°C. Mix thoroughly by inverting
9. Add the desired amount of Lipofectamine RNAiMAX to the OptiMEM. Prepare 10mL extra since this is the void volume of the tubing.
10. Mix the OptiMEM : RNAiMAX mixture by gentle pipetting with a pipette. This mixture is stable for 1hour at room temperature
11. Prime the WellMate with 20 ml of sterile PBS.
12. Enter 10 μ l as the volume to be dispensed in the WellMate dispenser and check that the plate format is 384 well.
13. Prime the WellMate with OptiMEM and RNAi Mix. (Be careful and do not discard too much mix during priming).
14. Immediately aliquot 10 μ l of the solution to each well. Centrifuge the plate @ 300 RPM ~30 sec and return to tissue culture biosafety hood.
15. Incubate this mixture for 30 minutes. The incubation should not exceed more than 60 min
16. Prepare the cells.

2.6.2 For cell culture—Calculate the number of cells required per day and prepare confluent flasks accordingly. Always have extra cells ready and prepare 80-100 ml excess of cell suspension.

1. Pre-warm DMEM-FBS-antibiotic growth media, 0.05% trypsin, and PBS without Ca/Mg.
2. Using the appropriate number of flasks, aspirate the growth media with 1 ml pipette. Add 10 ml PBS to each flask with 10 ml pipette. Aspirate the PBS with 1 ml pipette. Add ~1 ml of 0.05% Trypsin to each T-75 flask with 10 ml pipette. Return to the incubator for ~2min.
3. After trypsinization, add 9 ml DMEM-FBS-antibiotic growth media to each plate with 10 ml pipette and mix enough to ensure that all the HeLa cells are uniformly distributed. Pool and mix the cells.
4. Remove ~1ml of cell suspension for counting on automated cell counter
5. Calculate the number of cells and amount of cell suspension required keeping in mind 80-100 ml extra.

6. Prime the WellMate with 50 ml of sterile 1X PBS.
7. Mix the cells by gentle inverting the bottle.
8. Prime the WellMate with 20 ml of cell suspension.
9. Change the setting of the WellMate and use 35 μ l as the volume to be dispensed.
10. After T > 30min of incubation of the siRNA and lipid mix, add 35 μ l of DMEM containing 2000 HeLa cells to each well. **Do not centrifuge at this stage.**
11. Keep the plate inside the hood at room temperature for 5 min to allow uniform settling of cells.
12. Transfer the plates to humidified chambers in a 37 °C incubator. Incubate for 72 hours. It is advisable that the incubator should not be opened frequently to maintain humidity and minimize evaporation. We use Tupperware with wet paper towels as a cost-efficient humidified chamber.

2.6.3 Virus infection 3d post siRNA transfection

1. Pre-calculate the titer of virus and dilution/multiplicity of infection (MOI) during assay optimization. Prepare at least 30% or 15mL excess, whichever is larger.
2. Check at least one plate from the batch to make sure that the cell density is appropriate and that the cell death control killed all of the cells in the well.
3. Prepare the virus-media mix. Use DMEM+ 2% FBS + PKS.
4. Assemble the WellMate machine. Prime with 70% ethanol and keep it ready.
5. Prime the WellMate with 30 ml of sterile PBS.
6. Prime the WellMate with 20 ml of virus suspension.
7. Dispense 10 μ l of virus suspension to each well. If possible, a subset of wells should be left uninfected as a control.
13. Centrifuge the plates at 500 rpm for 5 min.
14. Prime the WellMate with 70% ethanol
15. Clean the hood.

2.6.4 Fixing and staining the cells post infection

1. Incubate the plate at 37°C in a humidified chamber for the optimized number of hours. We used 18 hours.
2. Prepare fresh 4% formaldehyde solution in PBS.
3. Aspirate the media from the infected cells using a multichannel aspirator inside the cell culture hood. Leave 5 μ l of media at the bottom of the well.
4. Prepare the WellMate dispenser. Prime it with 70% ethanol and then with PBS

5. Add 40 μ l of room temperature 4% paraformaldehyde in PBS to each well using the WellMate.
 6. Centrifuge at 300g for 1 min. Keep the plate at room temperature for 15 minute
 7. Set “Os wash program” in the Biotek plate washer.
 8. Wash the plates with PBS using the plate washer.
 9. Prepare PBS containing TritonX 100 and DAPI. Use WellMate dispenser and add 30 μ l Triton X 100 (0.1 %) to permeabilize the cells. Centrifuge for 30 sec and incubate for 20 minutes.
 10. Wash using Biotek plate washer with PBS.
 11. Use DAPI to counter stains the nuclei. Add 30 μ l of DAPI in PBS per well. Centrifuge and incubate for 20 minutes.
 12. Wash the plates using the plate washer.
12. Add 50 μ l of 1 X PBS using the multichannel pipette. Centrifuge the plates briefly. Seal the plates with plastic sealer and get ready for imaging.

2.6.5 Acquiring images using the ImageXpress Micro

1. Test the microscope using your pre-set settings optimized during the assay optimization and pilot screen.
2. Stack the plates in the robotic plate feeding system that can load the plates to the microscope. Load one plate. Activate barcode reader to register the plate name and ID.
3. Test settings with the first plate. Adjust as necessary. Save the protocol and image the entire plate. The images will be stored in the database.

2.6.6 Image analysis using MetaXpress software

1. Open the pre-saved analysis journal. Test settings with the first plate. Adjust as necessary to identify the DAPI and FITC positive cells.
2. Save the protocol and run for all the plates.

2.7 Visualization of screen data and quality control analyses

A clear SOP needs to be developed to allow for QC on each plate. This allows one to readily identify potential undesirable patterns or artifacts. Visualization of percent infection in a heat map format (Fig.5) as well as the performance of positive and negative control siRNAs should be monitored on each plate. Typically the genome-wide siRNA screen consists of >100 plates and is broken up and 10-30 plates are run per day according to the automation set up. The performance of control wells is also monitored each day to identify day-to-day variation. As shown in Fig.6 there is minimal day-to-day variation in the performance of the negative control samples. Heat maps and plate-well scatter plots to display overall screen performance and correlation between replicate screens is used to visualize overall reproducibility of the RNAi screen (Fig. 7). Cell viability as measured by cell number is also

important and should be determined using the above methods. Knockdown of essential cellular genes has cytotoxic effects and negatively influences cell number. Wells with significant toxicity should be analyzed separately and with care.

2.8 Statistical analyses and hit identification

The main objective of the primary siRNA screen is to identify ‘hits’ or ‘positive candidates’. This is basically the process of identifying which sample values differ meaningfully from those of the negative controls, or the mean/median of the population. The siRNA data are often normalized to remove systematic errors and the screen data from many plates can be combined. The z score, robust z score or B score can be used to normalize the data and identify candidates. This approach depends on hypothesis testing to determine the false positives and false negative rates of the assay hits. We typically use the z score (or robust z-score) is used to test the null hypothesis using the mean $\pm$ SD (or median $\pm$ k MAD (median absolute deviation)). Another approach is based on a rank list. For example, mean differences or folds changes are compared to control, and the sum rank, strictly standardized mean difference (SSMD) are calculated. For a more detailed review of these and other statistical approaches that have been applied to RNAi screens see (1, 2, 18-21). Ultimately, the selected candidate list forms the basis for subsequent validation.

2.9 Secondary assays

While a carefully optimized genome-scale primary screen can provide results of high confidence, a secondary screen is required to validate the primary screen results. Since many screens use a pooled approach, often the secondary screen deconvolutes the original pool using single siRNAs per gene. We recommend using independent siRNAs with distinct seed regions and not the same siRNAs from the screening set. Using single siRNA reagents per well and 3-4 siRNAs per gene allows one to set a threshold of at least 2 siRNAs per gene with the same phenotype as ‘true’ positives. However, if the secondary assay relies on the same readout as the primary assay the candidates may impact the assay but not viral infection. Therefore, we strongly recommend using a distinct assay for the secondary screen (eg, monitor infection by staining a viral antigen rather than a reporter). Furthermore, we suggest secondary screening in an independent cell line to avoid artifacts from transformed cells. This ‘validated’ gene set can be used for further study. We also recommend using orthogonal assays including CRISPR-mediated deletions, cDNA over expression, and small molecule inhibitors where possible, to strengthen the findings.

3 Discussion

The ability to harness the power of RNAi to perform loss of function screens has greatly contributed to our understanding of virus-host interactions. Table 1 lists some of the hurdles and caveats associated with screening which we will briefly discuss.

3.1 Factors contributing to the utility of the screen output

3.1.1 siRNA library design—The off-the-shelf siRNA libraries that are available can be obtained in a pooled format or in singles. The recent advances in siRNA design provide improved gene knockdown and reduced off target effects. The plate design should have

space for positive and negative controls to be spotted in each plate (Fig.4). If possible controls such as cell death control siRNA should be placed in each plate to verify the transfection efficiency. The primary screen should be conducted at least in duplicate to increase the statistical power for hit identification.

3.1.2 Cell line—For high content assays an adherent, readily transfectable (eg. HeLa, U2OS, A549), and infectable cell line should be used. Cells should be purchased from ATCC prior to screening, and should be mycoplasma tested. Cell stocks should be maintained to obtain a continuous supply of cells throughout the screening period.

The cell density is critical not only for virus infection but also dictates knockdown efficiency. The cell density should be optimized to obtain efficient gene knockdown and reproducible virus infection. During the assay optimization culture conditions should mimic the actual screen conditions.

The volume of cell suspension per well in a 384 well plates is also critical. Typically 50µl to 60µl of total volume is minimizes evaporation-mediated edge effects. Humidity should be maintained. We use a humid chamber inside the incubator and limit the traffic within the incubator.

3.1.3 Virus used and steps in the viral life cycle interrogated—Screens relying on microscopy are simplified through the use of a fluorescent reporter expressed from the virus of interest. This will reduce the workload involved in immunofluorescence staining and has low background.

The timing and MOI impacts the step of the virus lifecycle queried. For pro-viral factors, higher MOIs leading to higher percent infections are preferable while for anti-viral genes lower MOIs and lower percent infections are used. Moreover, the time post infection should be carefully chosen according to the step in the life cycle to be queried. For example, entry factors can be identified with short infections while longer infection times are required to study assembly or exit. Viral infection should be in the linear range so that genes impacting the step you are interested in can be readily identified.

3.1.4 Statistical methods—The statistical methods used to determine primary screen candidates play a major role in final candidate list determination (2, 18, 20, 21). Different types of statistical analyses can result in different gene sets (1). For example if the data is non-normally distributed a parametric method may not be used and a non-parametric method should be used. Rank lists have also been used with success (15, 17, 18, 20, 21). Different types of statistical analyses are suitable for hit selection in different situations. It is important to know the distribution of data to determine the appropriate statistical analysis.

Concluding remarks

Cell-based siRNA screening strategies have become a powerful tool to study virus-host interactions as well as other aspects of biology. Historically, weak silencing and off-target effects were problematic, but recent advances have improved RNAi reagents that present with more efficient gene knockdown and higher specificity. By optimizing the assay design,

data analysis and data interpretation RNAi screens have significantly increased our understanding of host-pathogen interactions. New information standards such as the MIARE (Minimum Information About an RNAi Experiment) and MIACA (Minimum Information about a cellular assay) guidelines are being recognized as a platform to present genome-scale data. Overall, RNAi screening is a versatile method to conduct genetic experiments in disparate cell types against diverse viruses. Moreover, RNAi screening can be integrated with diverse ‘omics’ technologies to expand our knowledge as well as potentially leading to new therapeutic avenues.

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Highlights

Genome-wide RNAi screen provides an extended platform to probe virus-host interactions

An overview of a complete siRNA screen for a RNA virus is presented

Arrayed format allows one to query the requirement for each gene product

This approach can be extended to other viruses and pathogens

Figure 1.
General work flow for a siRNA screen

Figure 2. Image analysis using MetaXpress. Images were captured using the DAPI and FITC channel. Image segmentation is shown for DAPI channel (DAPI positive cells are identified by blue circles) and FITC channel (GFP positive cells which are DAPI positive also are identified by green circles)

Figure 3.

Assay development using control siRNAs. Non-targeting (NT) siRNA and siRNA against a vATPase component (ATP6V0B1) was used to develop a siRNA screen. HeLa cells were transfected with NT and ATP6V0B1 siRNAs and subsequently infected with VSV. Cells were fixed and percent infection was analyzed. Transfection of NT has no effect on virus infection whereas knockdown of ATP6V0B1 reduces virus infection. This approach can be used to standardize siRNA knockdown efficiency, amount of transfection reagent required to obtain a phenotype and the Z' factor which is used to measure assay quality.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
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Figure 4.

Map of an assay plate. Plate map for a siRNA assay plate in 384 well format is shown. Test siRNAs are plated in columns 3 through 22 (shaded gray). Column 2 and 23 can be used for control siRNAs (shaded green). Column 1 or 24 can be used as a no infection control.

Figure 5. Heat map visualization of percent infection in a 384-well plate. HeLa cells were transfected and infected by VSV. Images were captured and analyzed for percent infection. Position of NT control siRNAs (well ID B2, B23, I2, I23) and positive controls restricting virus infection (D2, D23, F2, F23, J2, J23, L2, L23) is shown.

Figure 6. Day-to-day analysis of screen performance. The screen spans 11 days. Percent infection for negative control (no effect on virus infection) and positive control (which knocks down virus infection) wells are plotted for each day.

Figure 7.

Analysis of performance of control siRNAs in replicate screens. Scatter plot showing level of infection for negative control (blue diamonds; no effect on virus infection) and positive control (red squares; reduced virus infection) in two replicates. Positive control siRNA transfection leads to a strong reduction in virus infection as compared to the negative control siRNA treatment in replicate assays.

Reagents/parameters	Property/phenotype to consider
Virus	Reporter virus is ideal e.g. GFP expressing virus
Cell line	High transfection efficiency, adherent, robust viral infection
siRNA	Library should have low off-target effect, good knockdown efficiency with low concentration of siRNA. Typically 20-50 nM final concentration used.
siRNA format	Pool of 3-4 siRNAs per well targeting a gene is used. Should be run in duplicate
Negative control siRNA	No effect on assay readout such as virus infection, should not target human genes.
Positive control siRNAs	1. For transfection efficiency (e.g. Cell Death siRNA) 2. For viral replication (e.g. RAB5A siRNA).
Transfection reagent	Low cytotoxicity, Minimal effect on assay read out, high knock down
Transfection reagent incubation time with siRNA	Should have a large window to allow complex formation with the siRNA and to allow completing the reverse transfection procedure (typically 30-40 minutes is ideal).
Volume of cell suspension	Less volume uses less siRNA reagent and increases siRNA final concentration but more prone to evaporation effect/edge effect. Should be ideal to maintain the effective siRNA concentration with minimal effect on assay due evaporation.
Cell number	Cell number should be ideal for effective knockdown, virus infection. Overcrowding should be avoided. Cells should be evenly distributed across all wells in the plate.
Duration of knock down	Typically 48-72 hrs are required for knockdown. Should be optimized using the chosen cell line.
Percent of virus infection	Determine a range of infection where virus infection is linear. To identify the required factors percent infection is generally kept around 50%. To identify host restriction factors, percent infection is kept low, around 5-10%.