Chapter 4

Therapeutic Applications of RNAi for Silencing Virus Replication

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Abstract

RNA interference (RNAi) is an evolutionarily conserved gene-silencing mechanism in which small 19–23-nucleotide double-stranded RNA molecules, or small interfering RNAs (siRNAs), target cognate RNA for destruction with exquisite potency and selectivity. The RNAi machinery is believed to be expressed in all eukaryotic cells and has been shown to regulate host gene expression. Given this ability, RNAi silencing strategies have been developed to inhibit viral genes and replication in host cells. One area of growing interest is the development of synthetic siRNA drugs to target acute viral infections in which long-term gene silencing is not required or desirable. To achieve synthetic siRNA drug efficacy, these anti-viral agents need to be delivered to the appropriate host cells, as they do not readily cross the cell membrane. Varied delivery and siRNA chemical stabilization strategies are being investigated for siRNA drug delivery; however, several studies have shown that naked, unmodified siRNA drugs can be effective in silencing replication of some viruses in animal models of infection. These findings suggest that RNAi-based drugs may offer breakthrough technology to protect and treat humans and animals from viral infection. However, there are four major considerations for evaluating successful RNAi efficacy: the siRNAs must have high efficiency, show low cytotoxicity, result in minimal off-target effects, and lead to results that are reproducible between experiments. The methods and caveats to achieve these goals are discussed.

Key words: RNA interference, RNAi, small interfering RNA, siRNA, transfection, drug delivery, virus, prophylactic, therapeutic.

1. Introduction

RNA interference (RNAi) has been shown to be an important mechanism to specifically silence or downregulate gene expression. Key to the RNAi activity are small interfering RNA

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(siRNA) strands having complementary nucleotide sequences to cognate RNA targets. The highly selective and robust activity of siRNAs on gene expression has made RNAi a valuable tool for investigating gene function and regulating gene activity. Since synthetic siRNAs can be readily made and introduced into cells to mediate suppression of specific genes of interest, this technology is now routinely used to study gene function in cultured mammalian cells *(1)*, and to identify and validate potential drug targets in the host. However, RNAi has not been fully exploited as an anti-viral method, particularly for in vivo applications.

Use of RNAi in vivo has shown promise, and two basic methods for mediating RNAi have been used: delivery of siRNA *(2, 3)*, and delivery of plasmid and viral vectors that express short hairpin RNAs (shRNAs) that are processed in situ into active siRNAs *(2, 4)*. The field has predominantly focused on delivering siRNA because efficacy can be achieved by delivery across the cell membrane, whereas delivery across the nuclear membrane is not required, as it is for shRNA efficacy. Also, construction of shRNA expression vectors can be cumbersome, and substantially more time is required to test the efficacy of shRNA sequences. However, the success of siRNA-mediated gene silencing in vivo depends on delivery and maintenance of intact siRNA in the cells. Advances in this technology will be required to allow for highly efficient and specific siRNA delivery. Improvements in delivery methods will no doubt enhance the utility of the technique as a platform drug for disease intervention. Some progress has been made in siRNA delivery strategies that center on three general strategies: first, the development of novel siRNA conjugations and/or formulations to specifically target siRNA drugs; second, the development of siRNA carrier and/or encapsulation materials to protect siRNA drugs; and finally, optimal drug delivery methods, such as intravenous, aerosol, and topical *(2, 3)*. Given the drug-like properties of siRNA, and recent studies showing that naked or chemically unmodified siRNA duplexes may have robust virus gene-silencing activity in vitro and in vivo, there is good rationale and strong promise for therapeutic applications of RNAi for silencing virus replication.

2. Materials

2.1. Selection of siRNA Sequences for Targeting of mRNAs

1. The design of siRNA duplexes for targeting a specific gene requires knowledge of the target sequence. The decision to target specific viral genes may depend on factors such as

whether those genes code for receptors or affect the anti-viral immune response; however, a conservative approach would be to target transcripts from highly conserved genes critical in development of the virus nucleocapsid or involved in transcription of the virus genome, e.g., polymerase. For additional information refer to **Notes 1–13**.

2.2. Annealing of siRNAs to Produce Duplexes

1. Many commercial manufacturers, such as Dharmacon-ThermoFisher (Lafayette, CO), offer a range of pre-synthesized siRNA duplexes to meet the needs of the investigator; however, custom RNA synthesis may require an annealing step prior to testing.
2. To begin, prepare 2X annealing buffer (200 mM potassium acetate, 4 mM magnesium acetate, 60 mM HEPES–KOH, pH 7.4).
3. Prepare a 20 μM siRNA duplex working stock by combining 2X annealing buffer with sense oligonucleotide (20 μM final concentration) and antisense oligonucleotide (20 μM final concentration) in sterile water.
4. Incubate for 1 min at 90°C followed by 1 h at 37°C, and store the working stock solution at −20°C. The siRNA duplex solution can be freeze-thawed as required, but all RNA solutions should be kept on ice to reduce hydrolysis.
5. For quality control, the duplex solution should be run on a low-melt agarose gel in 0.5X TBE buffer at 80 V for 1 h, and the RNA bands subsequently visualized under UV light after ethidium bromide staining.

2.3. Selection of siRNA Transfection Tools

1. There are several approaches to RNAi but two are common for siRNA delivery: (a) lipid-mediated transfection of synthetic siRNA, and (b) viral-mediated transduction of siRNA. Determining which one of these approaches to use depends on the cell type studied and whether transient or stable knockdown is desired. For additional information refer to **Notes 14–25**.

2.4. Selection of Cell Lines for siRNA Screening

1. Various mammalian cell lines can be used for screening siRNA candidates, but Vero cells (ATCC No. CCL-81) or Vero E6 cells (ATCC No. CRL-1586) are preferred for reasons discussed in **Notes 26–29**.

2.5. SDS-PAGE Electrophoresis Reagents

1. 10X running buffer: 30 g of Tris base; 140 g of glycine; 10 g of SDS, all diluted in 1 l of distilled water.
2. Stacking gel buffer: 0.5 M Tris-HCL, pH 6.8; 0.4% SDS.
3. 2X solubilization buffer: 60 mM Tris-HCl, pH 6.8; 20% SDS; 0.0004% bromophenol blue; 10% mercaptoethanol; 20% glycerol.
4. Separation gel buffer: 1.5 M Tris-HCL, pH 8.8; 0.4% SDS.

5. 10X transfer buffer: 30.3 g Tris base; 144 g glycine, all diluted in 1 l distilled water; adjust pH with HCl to 8.3.
6. 10X TBS-T buffer: 24.2 g Tris base; 80 g of NaCl, pH 7.5; 10 ml of Tween-20, all diluted in 1 l of distilled water. Store at 4°C.

3. Methods

3.1. Evaluating siRNA Activity in Cells

1. Before siRNAs are applied, it may be important to establish if the cells to be studied are susceptible to RNAi, as it is possible that some cell lines have lost their ability to perform RNAi. This can be done using commercial reporter plasmids encoding firefly (*Photinus pyralis*) luciferase, GFP, or similar reporter molecules. Refer to **Notes 30–31** for additional cell type information.
2. To quantify siRNA concentrations that are unknown, the investigator should use Beer's Law: (Absorbance at 260 nm) = (epsilon)(concentration)(path length in cm), where epsilon is the molar extinction coefficient.
3. When solved for the unknown, the equation becomes: Concentration = (Absorbance at 260 nm)/[(epsilon)(path length in cm)]. When a standard 10 mm cuvette is used, the path length variable in this equation is 1 cm. If a different size of cuvette is used, e.g., a 2 mm microcuvette, then the path length variable is 0.2 cm.
4. Use the molecular weight of siRNA to convert between nmol and μg. If the specific molecular weight is not known, you may use the average molecular weight of siRNA, which is 13,300 g/mol. Therefore, 1 nmol siRNA = 13.3 μg, or 1 mg siRNA = 75 nmol.
5. In determining the quantity of siRNA that should be screened in an in vitro experiment, it is best to include a final concentration of range between 50–100 nM. For more information regarding preliminary in vitro screening refer to **Notes 32–37.**
6. Vero cells are ideal to begin in vitro studies. Vero cells should be maintained at the proper CO_2 in a humidified incubator at 37°C and grown in the appropriate media, e.g., Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS), but without antibiotics.
7. Sub-culturing is performed by passage using 0.25% (w/v) Trypsin in 0.53 mM EDTA solution using a sub-cultivation ratio of 1:3 with medium renewal twice per week.
8. For propagation and maintenance, warm the DMEM media and Trypsin-EDTA in a 37°C water bath

(Trypsin-EDTA made by diluting the stock 1:10 by adding PBS only).

9. Split the cells at 80–90% confluency by first decanting the media and washing the cells with PBS.
10. Aspirate off the PBS, add Trypsin-EDTA, and incubate at 37°C for approximately 3 min – be careful not to over incubate with Trypsin-EDTA as it will damage the cells.
11. Remove cells from the incubator and loosen the cells by gentle aspiration with a sterile pipette.
12. Place the dissociated cells in a sterile plastic tube with media containing 1% FBS to aid inactivation of the trypsin.
13. Count the cells using a hemacytometer or related device.
14. Dilute the cells in DMEM containing 10% FBS to an appropriate cell number for the vessel that will propagate them, usually about 1:5.
15. Repeat this procedure every 3–4 days, maintaining them twice a week so that they are not diluted too much or overgrown.
16. Refer to **Notes 38** and **39** regarding Vero cell storage and recovery.
17. For siRNA studies, 24 h prior to siRNA transfection, the cells should be trypsinized, washed in fresh DMEM medium without antibiotics, and aliquots transferred into each well of a 24-well plate. The Vero cells should be plated in 500 μl of medium without antibiotics so that that they will be approximately 50–70% confluent at the time of transfection.
18. For each transfection, prepare optimal siRNA/lipid transfection reagent complex as per the manufacturer's instructions. A dose–response should be performed with siRNA candidates and siRNA controls at concentrations ranging from 1 to 50 nM. Refer to **Note 40** for additional information.
19. Add the complex to the cells and gently mix by rocking the plate.
20. Incubate the plate at 37°C in a CO_2 incubator for 12–24 h. Transfection times may vary depending on siRNA stability, the siRNA sequence chosen, and the growth characteristics of the cell line being transfected.
21. If cell viability loss occurs, the transfection reagent–siRNA complex medium may be changed after 6–8 h.
22. Following transfection, the activity should be examined between 24 and 72 h siRNA. This can be done using a reporter-based assay (refer to **Notes 18** and **29**) that may be suitable for quantification by fluorescence-activated cell sorting, fluorescence, or as recommended by the manufacturer. It is important to note that transfection reagents change the autofluorescence of

cells, which must be controlled for by mock transfection.

23. For evaluating endpoints of siRNA activity in cells, refer to **Notes 41–50**.

3.2. Testing Viral Endpoints Following siRNA Treatment

There are several assays to evaluate siRNA efficacy against viruses, which fall into two main strategies, prophylactic or therapeutic application. Refer to **Note 51** for general information.

3.2.1. Virus Plaque Assay for Prophylactic siRNA Treatment Against Respiratory Syncytial Virus (RSV)

1. 24 h prior to siRNA testing, 24-well plates of the cell line of interest (e.g., Vero E6 cells) must be seeded to obtain 80–90% confluency.
2. Transfect the cells using a commercial transfection reagent, such as Mirus TransIT-TKO (TKO). Follow the manufacturer's transfection guidelines.
3. For example, for TKO-mediated transfection, add 2 μl of TKO to 50 μl of DMEM cell media that does not contain FBS and incubate at room temperature for 10 min.
4. Add siRNA (final optimal concentration needed is often ~100 nM) to TKO/DMEM mixture and incubate at room temperature for 10 min.
5. Remove tissue culture media from 24-h cultures of Vero E6 cells.
6. Wash gently with PBS and decant.
7. Add the siRNA/TKO mixture to cells and gently rock for 3 min.
8. Be sure to include controls: TKO only and scrambled or mismatched siRNA/TKO complex.
9. Incubate for 12 h (overnight) at 37°C, 5% CO_2.
10. At between 1–2 h post-siRNA transfection, remove plate from incubator and remove siRNA mixture from cells.
11. Prepare RSV virus (100–200 PFU/100 μl) in DMEM without FBS.
12. Add 100 μl of diluted RSV to appropriate wells of 24-well plate (test all siRNA concentrations in triplicate) and incubate for 1 h at 37°C, 5% CO_2.
13. At 2 h post-infection, remove plate from incubator and add 1 ml of DMEM/5% FBS containing 2% methylcellulose media to each well and incubate plate at 37°C, 5% CO_2 for 4–5 days.
14. At day 4 or 5 post-infection, remove overlay and visualize plaques by immunostaining with antibodies reactive to RSV F and/or G proteins, or counterstain, e.g., neutral red dye. Refer to **Note 52** for a word of caution about the assay.
15. To evaluate results, determine the percent plaque reduction from the controls, i.e., mismatched siRNA control and TKO control.

3.2.2. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and Quantitative Real-Time PCR (Q-PCR)

When using these methods to evaluate siRNA silencing of a gene, it is important to consider the interdependence between the targeting siRNA sequence and the PCR amplification region for assessing the efficacy of the siRNA target gene knockdown. Refer to **Note 53** for general information.

A generalized method for RT-PCR and Q-PCR is given below:

1. Total RNA is extracted from the transfected cells with RNeasy Mini Kit (QIAGEN).
2. Total RNA (2 μg) is reverse-transcribed to single-stranded cDNA using an oligo(dT) primer and ThermoScript RNase H^- reverse transcriptase (Invitrogen) according to the manufacturer's instructions.
3. Specific primers for the gene of interest must be prepared.
4. The RT-PCR reaction is carried out using the following mixture: 0.4 μl of cDNA template, 0.2 μl each of 1 mM gene-specific primers, 1.6 μl of 10 mM dNTPs, 2 U (0.4 μl) of Ex *Taq* (Takara Bio Inc., Siga, Japan), and 2 μl of 10 × PCR buffer.
5. Generally, 25 cycles of amplification are performed under the following conditions: 96°C for 30 s (denaturation), 30 s at 55°C (annealing), and 30 s at 72°C (extension). The PCR products are separated by electrophoresis in a 1.0% agarose gel containing 0.5 μg/ml ethidium bromide.
6. Q-PCR is carried out on a Real-Time PCR Detection System often using a QuantiTect SYBR Green PCR kit (QIAGEN). The Q-PCR reaction is carried out using the following mixture: 0.1 μl of cDNA template, 2 μl each of 1 mM specific primers, 25 μl of QuantiTect SYBR Green PCR Master Mix.
7. The Q-PCR amplification is performed under the following conditions: denaturation at 95°C for 15 min followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 30 s.
8. After Q-PCR, a melting curve is constructed by increasing the temperature from 55°C to 95°C.
9. Each sample for three independent experiments is run in quadruplicate. The mean cycle threshold (C_t) values for the gene are calculated and normalized to those for beta-actin. The mean value derived from each three experiments is calculated, and the results graphed with the corresponding standard deviation.

3.2.3. Western Blot Analysis

This is a method to detect a specific protein in the cell lysate using gel electrophoresis to separate native or denatured proteins by the length of the polypeptide (denaturing conditions) or by the 3-D structure of the protein (native/non-denaturing conditions). The proteins are then transferred to nitrocellulose or a

PVDF membrane where the protein can be detected, generally using antibodies. A general method for Western blotting is given below:

1. SDS-PAGE electrophoresis reagents are prepared as indicated.
2. To prepare 10% separation gel, mix 2.5 ml of 40% acrylamide/bis (9:1) solution, 2.5 ml of separation gel buffer, 5 ml of distilled water, 50 μl of ammonium persulfate, and 5 μl of TEMED. Cover the polymerizing gel by the layer of distilled water as it solidifies in the rig.
3. To prepare stacking gel, mix 1 ml of 40% acrylamide/bis (9:1) solution, 2.5 ml of stacking gel buffer, 6.5 ml of distilled water, 50 μl of ammonium persulfate, and 10 μl of TEMED. Dump out the layer of distilled water and layer the stacking gel on top of the separating gel.
4. To prepare sample, homogenize the particular material in solubilization buffer and incubate 30 min to 1 h at 40°C. Centrifuge sample for 2 min at 12,000 *g*.
5. Start electrophoresis in the pre-cooled running buffer at 70 V until the sample enters the separating gel, and then increase voltage up to 100 V.
6. To transfer the separated polypeptides onto the blotting paper, soak the blotting paper, e.g., Immobilon P cut to the size of the separating gel, in methanol for 2 min, and then accurately put the paper on the surface of water for 10 min. During this procedure the water replaces methanol from the paper pores.
7. Mix 100 ml of 10X transfer buffer with 850 ml of distilled water, cool the solution in the cold room, and add 50 ml of methanol.
8. Cut off the stacking gel and soak the separating gel in the prepared solution for 10 min at 4°C.
9. Soak two pieces of the Whatman 3MM paper with a size of separation gel in the prepared solution for 2 min.
10. Assemble sandwich, remove bubbles between gel and Immobilon P, and perform electrotransfer in the prepared solution for 1 h at 100 V.
11. Prepare 100 ml of TBS-T buffer. Add 5 g of dry nonfat milk. Shake vigorously.
12. Incubate the blotting paper in 15 ml of the prepared milk solution for 1 h at room temperature.
13. Wash the blotting paper two times with 15 ml of TBS-T buffer for 5 min.
14. Dissolve the primary antibodies in 15 ml of milk TBS-T buffer and add to the blotting paper. Incubate 1 h at room temperature.
15. Wash the paper with 15 ml of TBS-T buffer four times for 5 min.

16. Dissolve the secondary antibodies, e.g., conjugated to horseradish peroxidase, in 15 ml of milk TBS-T buffer and add to the blotting paper.
17. Prepare 6 ml of the substrate solution for horseradish peroxidase in accordance with manufacturer's protocol.
18. Incubate the blotting paper with this solution for 1 min. Wrap the wet paper with the Cling Wrap, put the surface of the covered blotting paper on film or imager, and detect.

3.2.4. Cell Cytotoxicity

Transfection-mediated cell cytotoxicity may be observed particularly during long-term exposure to transfection reagents. For general information refer to **Note 54**. The general protocol for performing cell cytotoxicity assays using the WST-1 method *(5)*, a highly sensitive and easy method, is given below:

1. WST-1 reagent is added to supernatants collected from transfected and control untransfected cell lines, i.e., 10 μl/100 μl of cell supernatant. This should be done in a 96-well plate format suitable for an ELISA plate reader.
2. The mixture is incubated for 0.5 h, and then the absorbance measured at wavelength 420–480 nm. Analyze the absorbance values to determine cell cytotoxicity compared to control wells.
3. Refer to **Note 55** for information regarding other commercial cytotoxicity kits.

3.3. Potential Pitfalls

There are important pitfalls to consider that may be encountered in prophylactic or therapeutic applications of RNAi. These include situations encountered in designing siRNA candidates, the host cell response to siRNAs, and evaluating efficacy. Refer to **Notes 56–64** for more information.

4. Notes

1. In considering target sites, intronic sequences should be avoided. Sequence information about mature mRNAs can be located from EST databases, e.g., www.ncbi.nlm.nih.gov.
2. It is important to compare the potential target sites to the appropriate genome database (examples given herein) to eliminate siRNA candidates with target sequences having more than 16–17 contiguous base pairs of homology to other coding sequences. Upon deciding on the target, the AUG start codon of the transcript should be scanned for AA dinucleotide sequences and the 3′ adjacent 19 nucleotides as potential siRNA target sites.

3. Since regions of mRNA may be highly structured or capped with regulatory proteins, several different siRNA target sites should be selected at different positions along the length of the gene sequence to ensure efficacy. It is important to note that the siRNA specificity for the target region is critical as a single point mutation is sufficient to abolish target mRNA degradation *(6)*.
4. Target sequences can be predicted from genomic sequences using prediction programs, e.g., exon.gatech.edu/GeneMark, genes.mit.edu/GENSCAN.html, or others.
5. In thinking about siRNA design, it is important to note that the most predominantly naturally processed and effective siRNAs have been shown to be duplexes of 21- and 22-nt with symmetric 2-nt 3′ overhangs *(6, 7)*.
6. Standard selection rules for siRNA design include the following: (**a**) the targeted regions on the mRNA sequence should be located 50–100 nt downstream of the start codon (ATG); (**b**) one should search for the sequence motif $AA(N_{19})TT$ or $NA(N_{21})$, or $NAR(N_{17})YNN$, where N is any nucleotide, R is purine (A, G), and Y is pyrimidine (C, U); (**c**) avoid sequences with >50% G+C content, and avoid stretches of four or more nucleotide repeats; (**d**) generally avoid targeting 5′ UTR and 3′ UTR regions; and (**e**) avoid sequences that share a certain degree of homology with other related or unrelated genes *(7)*.
7. Many commercial sites exist where one can send in the target gene information, and, using proprietary algorithms, the companies will develop synthetic siRNAs or siRNA pools for testing.
8. The siRNAs chosen for commercial design should be 19–23 nt in length and chemically synthesized using ribonucleoside phosphoramidites.
9. A typical 0.2 μmol-scale RNA synthesis generally provides about 1 mg, sufficient for approximately 1,000 in vitro transfection experiments using a 24-well tissue culture plate format.
10. There are a number of siRNA synthesis options including water-soluble, 2′-O-protected RNA which can be deprotected in aqueous buffers prior to use; fully deprotected RNA; and purified siRNAs duplexes. For most applications, the third option is preferred.
11. It is critical that the siRNA-targeted region on the mRNA sequence does not share significant, if any, homology with other genes or sequences in the genome, in order to minimize off-target effects.
12. It is preferable to select target regions such that siRNA sequences may contain uridine residues in the 2-nt

overhangs that can be replaced by 2′-deoxythymidine without loss of activity. This feature reduces costs of RNA synthesis and appears to enhance nuclease resistance.

13. It is advisable to synthesize siRNA specificity controls by scrambling the sequence of the effective siRNA duplex.
14. The most popular application for transient transfection of siRNA is the use of cationic lipid-based reagents because they are generally effective for delivering siRNA in many commonly used cell lines. There are many commercial suppliers of lipid-based transfection reagents that include Lipofectamine 2000™ (Invitrogen Corporation Carlsbad, CA) and Transit-TKO™ (Mirus Bio Corporation, Madison, WI).
15. Although lipid-based transfection is one of the more commonly used methods for adherent cells, suspension cells are often more difficult to transfect and generally have higher rates of delivery with electroporation techniques.
16. Although siRNA transfection using lipid-based reagents is a transient event, maximal efficacy is typically achieved 24–48 h after transfection, and may last for hours to days depending on the efficiency of transfection and the cell type being treated. A typical transfection protocol includes plating the cells, creating transfection complexes by mixing transfection agent with siRNA, and adding the transfection mixture to the growth medium with the cells.
17. shRNA can be expressed from lentivirus vectors allowing for high-efficiency siRNA transfection of a variety of cell types, including non-dividing cells and differentiated neurons of the brain *(8, 9)*. Approaches for lentiviral hairpin-mediated RNAi are numerous, as are methodologies for deriving RNAi-transgenic cells.
18. There are a multitude of commercially available lentivirus RNAi vectors, available at various websites, e.g., www.systembio.com, www. dharmacon. com, www.genscript.com, www. invitrogen.com, and others. Many of these vectors encode green fluorescent protein (GFP) or other markers for assessing infectivity, while other vectors are available with antibiotic selection markers such as puromycin. In addition, there are conditional lentiviral RNAi vectors available, with which the user can turn silencing on or off as required during the course of experimentation.
19. If a lentivirus RNAi vector must be constructed, an important first step is to design an effective RNA hairpin construct. Rules have been developed to enrich for successful hairpin constructs based on thermodynamic

analysis of siRNA efficacy. There are several prediction algorithms available for designing hairpin RNA, e.g., www-lbit.iro.umontreal.ca/mcfold/index.html and others, and several institutions have created lentiviral RNAi libraries that are available, e.g., mcmanuslab.ucsf.edu/.

20. Creating hairpin RNAi vectors can be accomplished by different methods. One approach involves annealing two long complementary DNA oligomers that are directionally cloned into the lentivirus expression vector. In a more involved approach, the entire hairpin sequence is included as part of one of the oligomers where the 300-nt U6 promoter is to be amplified by PCR and the resulting product contains the hairpin entity. These two approaches require long oligomers, thus often the final constructs are subject to sequence errors. To overcome this, other methods have been developed such as the synthesis of four different DNA oligomers whereby the oligomers are annealed and directly cloned into the lentivirus vector.
21. Since off-targeting of siRNAs generated by the lentivirus vectors may occur, it is advised that at least two different hairpin vectors should be made for each targeted gene. Thus, the vectors could be tested to determine if they have the same phenotypes.
22. To prepare a competent lentivirus from the DNA vectors, the DNA vectors are transiently transfected into a packaging cell line such as human 293 cells, and after 2–3 days the supernatant will contain the virus. These production systems generally incorporate a spilt-system, where the lentiviral genome has been split into individual helper plasmid constructs which diminishes the risk of creating a replication-capable virus by adventitious recombination of the lentiviral genome.
23. It is important to consider if the lentiviral vectors being prepared should have a restricted or broad host range. This feature is generally determined by the pseudotype or virus coat used – a feature that can be easily regulated as the lentiviral production systems are split. To generate a broadly tropic vector, one may consider using the vesicular stomatitis virus (VSV) glycoprotein, as opposed to another binding surface protein which would display limited host cell specificity.
24. After transfection of the 293 cells or other cell types has been achieved, the virus in the supernatant may be separated from the cells by filtration of the supernatant through a 0.45-μm syringe-filter. It is important to determine the virus titer in the supernatant so that experiments can be reproduced over the course of multiple experiments.

25. Different cell types differ in their ability to be infected. Polybrene (hexadimethrine bromide), a cationic polymer, may be used to increase the efficiency of infection of certain cells, as it acts by neutralizing the charge repulsion between the virions and cell surface.
26. As some RNA duplexes may stimulate type I IFN expression upon transfection into certain cell types, it is important to consider if a type I IFN-defective cell line can be used in the screening process, such as Vero or Vero E6 cells, a continuous line of African green monkey kidney cells defective in the production of interferon. This feature is particularly important for screening siRNA candidates that target viruses, as induction of type I IFNs can inhibit virus replication *(10–12)*.
27. It is important to maintain healthy cells. Some cell lines are more sensitive to transfection agents than others, and this may be dependent upon passage number. It is advisable to use cells subjected to a similar number of passages to ensure reproducibility in transfection results between experiments.
28. It is recommended that control transfections be performed by varying cell confluency (40–90%), as low cell density or too much transfection reagent increases cell toxicity.
29. To estimate the transfection efficiency in a particular cell line, it is recommended that fluorophore-conjugated siRNA be tested prior to experimentation, e.g., Cy3-labeled siRNA or fluorescein-siRNA transfection controls.
30. For studies involving siRNAs targeting viral genes it is often best to screen in vitro using flat-bottom 24-well plates as this allows for enumeration of virus titers as an endpoint.
31. For these type of studies, Vero or Vero E6 cells are preferred, but other cell lines have been used, including HeLa, COS, NIH/3T3, HEK 293, CHO, A549, and Drosophila cells *(13–15)*.
32. In preliminary in vitro siRNA screenings, the cells should be transfected as indicated by the manufacturer using a reliable transfection agent, e.g., Lipofectamine 2000 (Invitrogen).
33. The optimal ratio of siRNA-to-lipid transfection reagent and the total amount of this complex should be determined using the same number of Vero cells to evaluate the highest transfection efficiency.
34. Optimal ratios should be approached by starting with a constant amount of siRNA and varying the amount of transfection reagent. The best place to start is following the manufacturer's recommendations as each transfection reagent is different.

35. Reduced-serum medium is recommended for transfection; however, no antibiotics should be added to the media during transfection.
36. It is important to maintain the same cell numbers and conditions across experiments.
37. The serum-free media to be used in the assay should be tested for compatibility with the transfection reagent. Opti-MEM® is generally a good media to start trials with.
38. It is best not to exceed a passage number of 30 after thawing the stock culture, so make a master freezing of low passaged cells as the number of passages may affect siRNA transfection efficiencies.
39. Aliquots of cells of low passage number cells may be stored frozen by centrifuging the cells in a sterile tube and resuspending in a 9:1 ratio of FBS:DMSO. The cells should be aliquoted into appropriate freezer vials, put into Mr. Frosty containers first for freezing at –70°C and then moved into liquid nitrogen.
40. To transfect the cells with the siRNA candidates, it is best to use the conditions recommended by the manufacturer for the transfection reagents being used as they operate differently. For example, INTERFERin™, Dharma*FECT*™, HiPerFect™, Transit-TKO™, Lipofectamine 2000™, and other transfection reagents are available for siRNA transfection of eukaryotic cells, especially at low siRNA concentrations, e.g., 1–10 nM siRNA concentrations.
41. Optimally, siRNA should be delivered to the cells 12–24 h prior to virus infection for siRNA drugs targeting viruses. Endpoints such as virus titers are generally assessed 48 h post-transfection. It is important not to overwhelm the cells during the infection phase; multiplicities of infection (MOIs) between 0.1 and 1.0 are generally used.
42. siRNAs targeting different positions on the same gene or different genes may induce different levels of gene silencing. Therefore, the selection of the mRNA target sequence is critical to siRNA efficacy. Equally important are the endpoints for analysis of siRNA efficacy. For virus studies these endpoints may include examining virus titer, qPCR of virus gene expression, plaque morphology, viral antigen levels, or loss of reporter signal such as that associated with a GFP-virus.
43. Effective siRNA knockdown of virus replication can be deduced by determining the reduction of virus titer, or at least demonstrating a reduction of the targeted viral mRNA. An immunostaining virus plaque assay using monoclonal antibodies specific to a viral protein will allow for quantitation of virus replication.

44. Standard plaque assays using counterstaining may also be effective, but for this and immunostaining plaque assays, it is recommended that a commercial plaque counting device be used to minimize investigator error. ELISPOT plate readers such as the AID EliSpot Reader has a software program that counts spots according to the user's settings (www.elispot.com/index.html?elispot_reader/software.htm).
45. It may be useful to examine expression of the targeted viral protein by Western blotting of virally infected cell lysates.
46. If no siRNA knockdown of protein is observed by Western blot, it may be desirable to analyze whether the target mRNA was effectively destroyed by the transfected siRNA. This can be tested by preparing total RNA from the transfected and mock-treated controls between 24 and 72 h post-transfection.
47. The total RNA can be reverse-transcribed using a target-specific primer and PCR-amplified with a primer pair covering at least one exon–exon junction in order to control for amplification of pre-mRNAs.
48. RT-PCR of a non-targeted mRNA is also needed as control. Effective depletion of the mRNA with undetectable reduction of target protein may indicate that a large reservoir of stable protein exists in the cell. Multiple transfections in sufficiently long intervals may be necessary until the target protein is finally depleted to a point where a phenotype may become apparent.
49. If multiple transfection steps are required, it is recommended that the cells be split every 2–3 days after transfection. The cells may be transfected immediately after splitting. Note that cells diluted to a confluency of less than 60% are generally less effectively transfected.
50. If detection of siRNA-mediated knockdown cannot be observed, test a different siRNA duplex targeting a different gene region or gene, test for the possibility of sequencing errors in deposited sequence files, or for polymorphisms that may be a problem for a given sequence. It is recommended that that the cell line used be investigated, as ~36% of cell lines are of different origin or species to that claimed *(16)*.
51. Prophylactic treatment is the most common method used to evaluate efficacy, where the siRNAs are delivered to the cells of choice prior to virus infection. Therapeutic treatment is done after virus infection of the cell line. In both cases, virus plaque assays are commonly used to evaluate efficacy. A plaque assay measures the number of infectious virus particles. The methods for these assays vary and are dependent upon the virus being studied, as viruses

exhibit cell tropism, cell receptor requirements, cell enzyme requirements, etc. It is important to be familiarized with the current literature for the appropriate methods to use for the virus to be studied.

52. A word of caution – HEp-2 cells (ATCC # CCL-23) are also commonly used to propagate and titer RSV. It is important to note that this line was originally thought to be derived from an epidermoid carcinoma of the human larynx, but was subsequently found based on isoenzyme analysis, HeLa marker chromosomes, and DNA fingerprinting, to have been established via HeLa cell contamination. Thus, these cells are not recommended for use.
53. Accurate measurement of siRNA efficacy by PCR requires careful attention to the primer location used to amplify the target mRNA. It is known that initial cleavage of the target gene mRNA by the RNA-induced silencing complex (RISC) endonuclease occurs near the center of the siRNA targeting sequence *(6)*, an effect mediated by Ago-2 activity *(17)*; however, complete degradation of the entire mRNA is not always observed *(18, 19)*.
54. Some siRNAs have been reported to induce off-target effects linked to cell cytotoxicity, an effect that may obscure evaluation of siRNA efficacy. There are a number of commercially available kits for evaluating cell cytotoxicity. The WST-1 method *(5)* is highly sensitive and easy to perform. WST-1 is a tetrazolium salt that when in contact with metabolically active cells gets cleaved to formazan by mitochondrial dehydrogenases. The formazan dye is then measured using a scanning spectrophotometer at wavelengths 420–480 nm. The darker the formazan dye, the greater the number of metabolically active cells in that well. The WST-1 reagent has several advantages compared to other cell cytotoxicity reagents in the market. The WST-1 reagent is water-soluble after cleavage so there is no need to perform a solubilization step, and WST-1 is very stable so it can be stored in a ready-to-use solution.
55. There are also commercial kits that provide a direct measurement of cytotoxicity rather than using an indirect indicator such as the release of cell enzymes. An example of a kit is given by the LIVE/DEAD® Viability/Cytotoxicity Assay Kit from Molecular Probes. This kit provides a two-color fluorescence cell viability assay that is based on the simultaneous determination of live and dead cells with two probes that measure two recognized parameters of cell viability: intracellular esterase activity and plasma membrane integrity. The kit can be used with fluorescence microscopes, fluorescent plate scanners, or flow cytometers. The assay is applicable to most eukaryotic cell types, including

adherent cells. It is recommended that the user follow the manufacturer's protocol as it varies depending upon application.

56. When designing siRNA candidates it is important to note that if the number of allowed mismatches between siRNA and homologous gene sequences are set too stringently, some functional siRNAs will be rejected due to sequencing errors that are common to EST databases.
57. Several studies have shown that the host cell interferon-response effect is enhanced by increasing the concentration of siRNAs *(11, 12)*.
58. siRNA duplexes >23 bp can influence cell viability and induce a host cell IFN response that appears to be mediated through upregulation of the dsRNA receptor, Toll-like receptor 3 *(20)*. These effects may be cell type specific.
59. Several studies have shown that off-target effects may be caused by perfect matches of the seed region of the siRNA antisense strand with the 3′ untranslated region of unintended mRNA targets, combined with limited homology elsewhere in the 21-base target sequence *(21, 22)*.
60. Due to the risk of off-target effects, it is critical to design siRNA experiments with redundancy so that spurious results can be detected. Redundancy experiments should use multiple different silencing reagents, e.g., several siRNAs targeting different areas of the same mRNA. As the probability that several siRNAs that target different sequences of the same gene would cause the same phenotype through off-target interaction is very low, this approach can be used to show siRNA specificity and the validity of the relationship between target gene knockdown and resulting phenotype.
61. In designing short siRNAs, it is important to consider that they may target a part of the mRNA that is masked by secondary structure or bound proteins, thus reducing efficacy through inaccessibility of the target site.
62. A negative siRNA screening result may be due to inefficacy that could result from an inability to reduce translation to the point where a phenotype is apparent.
63. With regard to the therapeutic window of siRNA efficacy, it is important to consider the level of siRNA uptake and intracellular processing by the cell type being investigated.
64. If the goal is siRNA therapeutic approaches, it is important to consider the breadth of applicability to the clinically relevant target, the level of cell cytotoxicity associated with delivery of the siRNA, and other regulatory perspectives.

    Another limitation to consider is that clinical use of siRNAs would typically be transient in nature with

intracellular siRNA concentrations and efficacy usually declining within a 1–2 week period. Thus, treatment of chronic pathologies would either require repetitive siRNA treatment, or long-term RNAi linked to stable transfection, e.g., lentivirus vectoring.

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