Chapter 17

Design of Small Interfering RNAs for Antiviral Applications

Diana Rothe, Erik J. Wade, and Jens Kurreck

Abstract

RNA interference (RNAi) is an evolutionarily conserved mechanism for sequence-specific target RNA degradation in animals and plants, which plays an essential role in gene regulation. In addition, it is believed to function as a defense against viruses and transposons. In recent years, RNAi has become a widely used approach for studying gene function by targeted cleavage of a specific RNA. Moreover, the technology has been developed as a new therapeutic option that has already made its way into clinical testing.

Treatment of viral infections remains a serious challenge due to the emergence of new viruses and strain variation among known virus species. RNAi holds great promise to provide a flexible approach that can rapidly be adapted to new viral target sequences. A major challenge in the development of an efficient RNAi approach still remains the design of small interfering RNAs (siRNAs) with high silencing potency. While large libraries with validated siRNAs exist for silencing of endogenously expressed genes in human or murine cells, siRNAs still have to be designed individually for new antiviral approaches. The present chapter describes strategies to design highly potent siRNAs by taking into consideration thermodynamic features of the siRNA, as well as the structural restrictions of the target RNA. Furthermore, assays for testing the siRNAs in reporter assays as well as options to improve the properties of siRNAs by the introduction of modified nucleotides will be described. Finally, experimental setups will be outlined to test the siRNAs in assays with infectious viruses.

Key words: Cell viability assay, Plaque assay, RNA interference, shRNA, Short hairpin RNA, Small interfering RNA

1. Introduction

Despite the advances in medical sciences, the threat posed by viruses has remained a serious problem, particularly outside industrialized countries. While the human immunodeficiency virus (HIV) and the hepatitis B and C viruses (HBV, HCV) continue to claim millions of lives every year, viruses like the SARS coronavirus appear as human pathogens and novel variants of

well-known viruses, like the H1N1/09 influenza virus, cause pandemics of global dimensions. Thus, there is a pressing unmet medical need to develop new antiviral drugs. While small molecules have dominated antivirals for decades, biologics like monoclonal antibodies and nucleic acids therapies have more recently been considered promising alternatives (1).

Among the various oligonucleotide-based strategies, RNA interference (RNAi) is widely regarded as a particularly powerful technology (2–4). RNAi is a posttranscriptional gene silencing mechanism that is triggered by double-stranded RNA. For applications in mammalian cells, small interfering RNAs (siRNA) of approximately 19 base pairs with two nucleotide overhangs at the 3′ ends of both strands become incorporated into the RNA-induced silencing complex (RISC) and induce cleavage of a complementary target RNA. Within just a few years, RNAi has become a standard molecular biological method to study gene functions. Moreover, the technology has been developed into a new therapeutic approach and approximately a dozen clinical trials based on RNAi are currently underway. Among these trials are various applications of RNAi to treat infections with the respiratory syncytial virus, HBV and HIV (5).

Silencing of endogenously expressed genes has become a routine procedure and predesigned or validated siRNAs with silencing guarantee against virtually any human or murine gene are commercially available. In contrast, the design of siRNAs with high antiviral activity still remains a challenging task. Several features have been described that influence the success of an RNAi approach (6):

- Thermodynamic design of the siRNA.
- Structure of the siRNA antisense strand.
- Structure of the target RNA.

Most of the publically available tools for the design of siRNAs exclusively optimize the base composition of the siRNA. More recently, however, a design algorithm was developed for the selection of siRNA with particularly high potency and specificity, which not only focuses on the design of the siRNA, but also takes into consideration the secondary structures of the siRNA and their target site (7).

A major challenge for the long-term inhibition of viruses by RNAi is the prevention of viral escape. Thus, siRNAs need to be directed against well-conserved target sites. Unfortunately, simply directing siRNAs to protein coding regions is insufficient, since silent mutations can cause the siRNAs to lose their inhibitory potential. Target regions with an important function in the structure of the RNA may prove to be a better choice to avoid viral escape. It has, for example, been shown that an siRNA against

the highly conserved cis-acting replication element (CRE) was capable of inhibiting various enteroviruses over a long period, while an siRNA targeted against protein-encoding regions of the RNA, which are not organized into functional three dimensional structures, led to rapid viral escape (8). Since even the most careful selection of a single target site might be insufficient for sustained viral silencing, use of multiple siRNAs against multiple target sites may be necessary to prevent viral escape. In the case of coxsackievirus B3 and HIV-1, resistance rapidly emerged when single molecules were used, but a combination of three or four siRNAs, respectively, targeting distinct regions of the genome, were able to prevent the emergence of resistance (9, 10).

A general requirement for the application of RNAi in vivo is the stabilization of siRNAs by the introduction of modified nucleotides (11). Based on the experience in the antisense field, numerous modified nucleotides have been assessed for their applicability to enhance the stability of siRNAs against nucleases. Phosphorothioates, nucleotides with modifications at the 2′ position (e.g., 2′-O-methyl-RNA and 2′-fluoro-nucleotides), as well as locked nucleic acids are among the most widely employed building blocks for RNAi applications. A fully modified siRNA with a drastically increased half-life in human serum had a significantly higher activity in a vector-based in vivo model of HBV infection as compared to the unmodified form (12). Furthermore, the introduction of modified nucleotides not only improves the stability of siRNAs, but can also reduce off-target effects (13) and improves the antiviral activity of siRNAs directed against highly structured target regions (14).

The present chapter describes a systematic method for the design of highly potent antiviral siRNAs (summarized in Fig. 1). Experimental procedures to test these siRNAs in reporter assays (GFP Reporter Assay and Dual-Luciferase Reporter Assay) as well as in assays with infectious viruses (Cell viability assay and Plaque reduction assay) will be outlined. Finally, options to improve the properties of siRNAs by introduction of modified nucleotides will be described.

2. Material

2.1. GFP Reporter Assay

1. CT- or NT-GFP Fusion TOPO TA Expression Kit (Invitrogen, Carlsbad, CA, USA). Store the competent TOP10 cells at −80°C, and all other components of the kit at −20°C.

2. Materials for isolation of viral DNA or RNA. We recommend use of a commercial isolation kit, for example, the High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Mannheim, Germany).
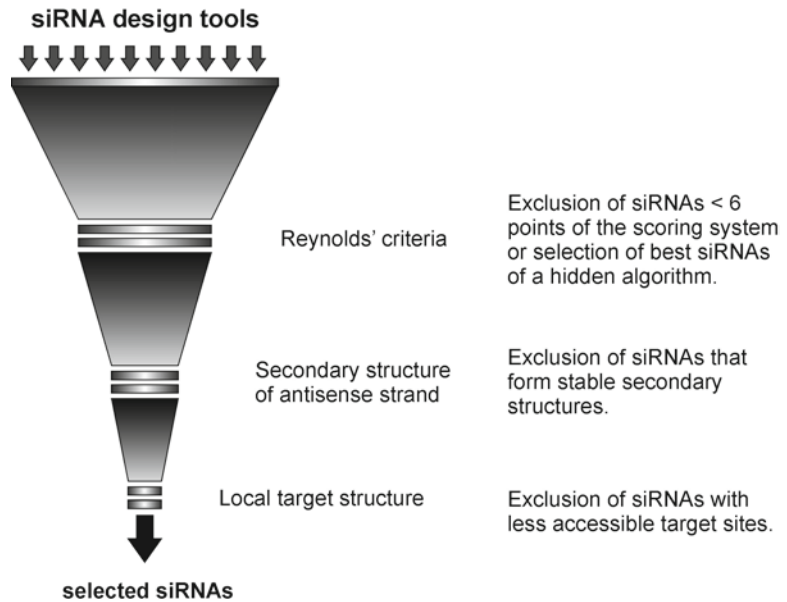
Fig. 1. Systematic selection of antiviral siRNAs. In the first step, siRNAs are scored according to a system based on the thermodynamic features of the siRNA (15). Next siRNAs with antisense strands that are predicted to form stable secondary structures are excluded. Finally, the accessibility of the target site in the viral RNA is tested to end up with a pool of siRNAs which are suitable for testing in vitro.

3. Materials for reverse transcription if the genome of the isolated virus is RNA.

4. Materials for PCR and *Taq* polymerase if a polymerase with proofreading activity is used in PCR.

5. Materials for the Gel purification of DNA. We recommend use of a commercially available gel purification kit, for example, the NucleoSpin Extract II (Macherey-Nagel, Düren, Germany).

6. LB medium: Dilute 1% (w/v) Tryptone, 1% (w/v) NaCl, and 0.5% (w/v) yeast extract in H_2O and autoclave to sterilize. LB Agar plates additionally contain 1.5% (w/v) Agar. For selection, add 100 µg/ml ampicillin. Store LB medium and LB Agar plates at 4°C, but warm to room temperature before use.

7. Materials for the isolation of plasmid DNA. For test-restrictions and sequencing, any DNA Plasmid Miniprep (Kit) is sufficient. For transfection-grade plasmids, we recommend to use a commercially available Plasmid DNA Isolation Kit, for example, the NucleoBond Xtra Midi Kit (Macherey-Nagel).

8. Primer for sequencing of pcDNA3.1/CT-GFP-TOPO: T7 (forward): 5′-TAA TAC GAC TCA CTA TAG GG-3′ and

GFP Reverse (reverse): 5′-GGG TAA GCT TTC CGT ATG TAG C-3′.

9. Primer for sequencing of pcDNA3.1/NT-GFP-TOPO: GFP Forward (forward): 5′-CGA CAC AAT CTG CCC TTT CG-3′ and BGH Reverse (reverse): 5′-TAG AAG GCA CAG TCG AGG-3′.

2.2. Dual-Luciferase Reporter Assay

1. psiCHECK-2 Vector (Promega, Madison, WI, USA). Store at –20°C.

2. The same materials which are described for GFP Reporter Assay (see Subheading 2.1) except items 1, 8, and 9.

3. Restriction enzymes Not I and Xho I. We recommend use of commercially available restriction enzymes with optimized buffer solutions.

4. T4 DNA Ligase. We recommend use of a commercial DNA ligation kit with a corresponding buffer.

5. Primer for sequencing of psiCHECK-2 vectors: Reverse primer: 5′-CTC ATT TAG ATC CTC ACA C-3′.

6. Dulbecco's Phosphate-buffered salt solution (PBS) without Ca and Mg (PAA Laboratories GmbH, Cölbe, Germany).

7. Dual-Luciferase Reporter Assay System (Promega). Store all components of the Kit at –20°C. For long-term storage, prepare aliquots and store at –70°C.

8. Luminometer.

2.3. Cell Culture and Transfection

1. Dulbecco's modified Eagle's medium (DMEM) (PAA Laboratories) supplemented with 10% fetal calf serum (FCS) (PAA), 2 mM glutamine, non-essential amino acids.

2. Dulbecco's PBS without Ca and Mg (PAA Laboratories).

3. Opti-MEM (Invitrogen) or serum-free DMEM.

4. Lipofectamine 2000 transfection reagent (Invitrogen). Store at 4°C.

5. Fluorescence microscope with GFP filter and additional Cy3 filter if the transfection efficiency of siRNAs into other cell lines has to be tested.

2.4. Lysis and Western Blot

1. Dulbecco's PBS without Ca and Mg (PAA).

2. DTT Lysis buffer: 62.5 mM Tris–HCl, pH 6.8, 2% (w/v) SDS, 50 mM DTT, 10% (v/v) glycerol, and 0.05 (w/v) bromophenol blue. Store in aliquots at –20°C.

3. Tris-buffered saline (TBS): 20 mM Tris, 150 mM NaCl, pH 7.5. Prepare a tenfold stock solution containing 200 mM Tris and 1.5 mM NaCl. Adjust to pH 7.5 with HCl and store at room temperature. For 1× TBS, dilute the stock solution 1:10.

4. TTBS: Prepare a solution containing TBS and 0.1% Tween 20. Store at room temperature.

5. Materials for standard western blot including Hybond-P PVDF membranes (Amersham/GE Healthcare, Uppsala, Sweden) and non-fat dry milk powder.

6. Ponceau solution: 0.2% Ponceau S, 3% acetic acid in water. Store at room temperature.

7. Primary antibodies: Polyclonal Anti-GFP rabbit serum (Invitrogen), store at –20°C. Use at a 1:5,000 dilution in western blot. Mouse monoclonal anti-Actin antibody (Chemicon/Millipore), store at 4°C. Use at a 1:5,000 dilution in western blot.

8. Secondary antibodies: Immuno Pure Goat Anti-Rabbit IgG (H + L), Peroxidase Conjugated (Pierce, now part of Thermo Fisher Scientific, Rockford, IL, USA). Immuno Pure Goat Anti-Mouse IgG (H + L), Peroxidase Conjugated (Pierce). Store at –20°C.

9. ECL Western blotting substrate (Pierce).

10. Chemiluminescence Imager or material for wet development inclusive X-ray films.

2.5. Cell Viability Assay

1. Materials for siRNA transfection (see Subheading 2.3, items 1–4).

2. Virus stock of interest.

3. Cell Proliferation Kit II (XTT) (Roche Diagnostics). Store both the XTT labeling reagent and the electron-coupling reagent in small aliquots at –20°C and protect from light.

4. ELISA plate reader for measurement of absorbance at a defined wavelength between 450 and 500 nm, with a reference wavelength greater than 650 nm.

2.6. Plaque Reduction Assay

1. Materials for siRNA transfection (see Subheading 2.3, items 1–4).

2. Virus stock of interest.

3. Difco Agar Noble (Becton Dickinson, Franklin Lakes, NJ, USA).

4. Eagle's MEM: Dilute 9.53 g of Minimum Essential Medium (MEM) powder (Invitrogen) and 2.2 g $NaHCO_3$ in 500 ml water. Add antibiotics, penicillin, and streptomycin. Sterilize by filtration through a 0.2-μm filter. Store at 4°C.

5. Neutral red solution: Dilute 0.02 g neutral red (Merck) in 50 ml PBS and filter to remove undissolved particles. Avoid exposing the solution to light for longer than necessary. The dilution has to be prepared freshly.

3. Methods

3.1. Bioinformatics: siRNA Design

1. The first major step for the design of highly active siRNAs consists of the selection of siRNA sequences with favorable thermodynamic features. The thermal stability of both ends, as well as base preferences of active siRNAs at certain positions, influence the silencing efficiency of an siRNA (15). For the design of siRNAs, publically available web tools are used (examples are given in Table 1). It is advisable to generate an siRNA pool with at least two different web tools. The BLAST option should be applied to avoid unspecific binding to endogenous target sequences, thereby reducing off-target effects. As an example, siRNA predesign using *RNAi Designer* from Invitrogen is described below:

    (a) Go to the *RNAi Designer* website from Invitrogen (see Table 1).

    (b) Choose *siRNA* as "Target Design Options."

    (c) Enter the complete or partial target sequence. Use highly conserved regions of the virus (see Note 1).

    (d) Deselect all options for target region selection (ORF, UTR).

Table 1
List of web-based algorithms for the design of siRNAs

Source	ULR
Dharmacon	http://www.dharmacon.com/DesignCenter
Integrated DNA Technologies	http://www.eu.idtdna.com/Scitools/ Applications/RNAi/RNAi.aspx
Sonnhammer Lab	http://sirna.sbc.su.se/
Invitrogen	https://rnaidesigner.invitrogen.com/ rnaiexpress/
Qiagen	www1.qiagen.com/GeneGlobe/
Sfold Algorithm	http://sfold.wadsworth.org/index.pl
Whitehead Institute	http://jura.wi.mit.edu/bioc/siRNAext/ (requires registration)
MWG-Biotech	www.eurofinsdna.com (requires registration)
Applied Biosystems	http://www5.appliedbiosystems.com/tools/ siDesign/

    (e) Select the BLAST database (e.g., if human viruses are to be targeted, choose *human* to avoid target sites within the human genome; for initial testing in animal models, homology to the animal's genome should be avoided as well).

    (f) "GC content" should be set to a minimum *of 25%* and a maximum *of 55%*.

    (g) Select *default motif pattern* (see Note 2).

    (h) Submit by pressing the *RNAi Design* button. A summary of designed siRNAs is given in a new window. *Sort* the output by choosing *ranking*.

    (i) Select siRNAs with 4½ to 5 stars and copy the sequences.

    In general, choose the best siRNAs obtained with a design tool. If the siRNA design tool did not include a BLAST option, an NCBI BLAST should follow (see Note 3).

2. The obtained siRNA pool is subsequently filtered with respect to their thermodynamic features:

    (a) Convert all sequences to obtain the antisense strand of each siRNA in a 5′ to 3′ orientation. Antisense strands are complementary to their target RNA (see Fig. 2).

    (b) Remove duplicate siRNAs. Only 100% identical siRNA sequences should be excluded since a single nucleotide shift can sometimes influence the efficiency of an siRNA.

    (c) Calculate the score of each siRNA based on the scoring system given in Table 2.

    (d) Exclude siRNAs that have less than 6 points of Reynolds' criteria.

3. The second major step is to exclude siRNAs whose antisense strand is predicted to form secondary structures. To this end, the change in Gibbs free energy (ΔG) of the siRNA antisense strand is determined. Negative ΔG values indicate stable secondary structures, whereas positive ΔG values indicate instable secondary structures resulting in a high degree of unfolded


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DNA         5' ⌒⌒ gctgacatgaatgatctaa ⌒⌒ 3'

RNA         5' ⌒⌒ gcugacaugaaugaucuaa ⌒⌒ 3'

sense       5'      gcugacaugaaugaucuaadTdT    3'
                    ⁞⁞⁞⁞⁞⁞⁞⁞⁞⁞⁞⁞⁞⁞⁞⁞⁞
antisense   3'   dTdTcgacuguacuuacuagauu         5'
```
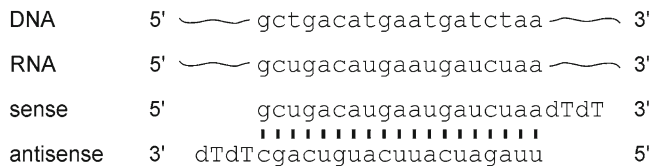
Fig. 2. Orientation of sense and antisense strands of an siRNA in relation to corresponding strands of DNA and RNA.

Table 2
Scoring system based on the thermodynamic features of the siRNA (according to Reynolds et al. (15))

Criterion	Score
GC content between 30 and 52% (in the 19mer siRNA duplex)	1 Point
A or U at positions 15–19	1 Point for each
Lack of internal repeats ($T_m < 20°C$)	1 Point
A at position 19	1 Point
A at position 3	1 Point
U at position 10	1 Point
G or C at position 19	–1 Point
G at position 13	–1 Point

Positions refer to the sense strand of the siRNA in 5′ to 3′ orientation

Table 3
Additional websites

Source	URL
Rensselaer bioinformatics web server	http://mfold.bioinfo.rpi.edu/
Burnet Institute mfold server	http://mfold.burnet.edu.au/
NCBI BLAST	http://blast.ncbi.nlm.nih.gov/Blast.cgi
Ensembl BLAST	http://ensembl.org/Multi/blastview/
Invitrogen	www.invitrogen.com (search for "GFP Fusion TOPO TA Expression Kit")
Promega	www.promega.com (search for "psiCHECK-2")
New England Biolabs	www.neb.com/nebecomm/tech_reference/restriction_ enzymes/cleavage_olignucleotides.asp
Turner energy rules	http://www.bioinfo.rpi.edu/zukerm/rna/energy/

single-stranded RNA. The ΔG determination using the publically available *UNAfold* software is described. This software extended and replaced the *mfold* software of Zuker (16).

(a) Go to the Rensselaer bioinformatics web server (see Table 3) and use the DINAMelt Web Server.

(b) Select the application *Nucleic Acid Quikfold*.

(c) Enter all siRNA antisense strand sequences (5′ to 3′) which were obtained after first selection step (thermodynamic design of siRNAs, steps 1 and 2) separated by semicolons.

(d) Choose *RNA* (*3.0*) in the "energy rules" field. The RNA is now folded at the fixed temperature of 37°C. The hybridization temperature can be modified by using *RNA* (*2.3*). Additionally, "sequence type" has to be specified as *linear*. Other settings in this window are not necessary.

(e) *Submit* to *DINAMelt Server*. A summary of potential folding results is given in a new window (see Note 4). Exclude all siRNAs whose antisense strand is predicted to form secondary structures indicated by at least one structure with a negative ΔG value. Structures with ΔG values around zero have a folding probability of 0.5 (17) and should be further analyzed (see Note 5).

4. The accessibility of the target RNA sequence will be considered in the third major step of siRNA design. The procedure consists of two steps: First, folding of the entire target RNA molecule and, second, the determination of the change in Gibbs free energy (ΔG) of the local RNA target site. ΔG determination using the publically available *mfold3.2* software, which should be used preferentially for long RNA sequences, is subsequently described (16).

(a) Go to the "Rensselaer bioinformatics web server" (see Table 3) and use the Mfold Web Server.

(b) Select the application RNA*fold*.

(c) Enter the "name" and the "sequence" to be folded.

(d) Choose "RNA sequence is" *linear*. The folding temperature is fixed at 37°C. This temperature can be modified by using the *RNA mfold version 2.3 server*. The "upper bond on the number of computing folding" can be limited to 10 or less for long sequences as they may increase the computational load and resulting response time. Other settings in this window are not necessary.

(e) Sequences shorter than 800 bases can be folded immediately (as of March 2010). For longer RNA sequences up to 6,000 bases, select *batch* and enter your *E-mail address*.

(f) Submit your entries by pressing the *Fold RNA* button. RNA sequences longer than 6,000 bases can be folded by the alternative "Burnet Institute mfold Server" (see Table 3). The application *Submit a RNA sequence* is based on *mfold version 3.0*. Parameters in this window

can be set according to the *mfold version 3.2* described above, except the folding temperature, which is not fixed at 37°C. Additionally, limit the "upper bond on the number of computing folding" to 10. Both "Rensselaer bioinformatics web server" and "Burnet Institute mfold Server" will give a summary of potential folding results.

(g) ΔG is calculated for a number of potential secondary structures (listed as "Structure 1, 2, 3 …" beneath "View Individual Structures"). The more negative ΔG, the more stable secondary structure is going to be, which also indicate a higher chance of its presence in a biological system.

(h) Save at least ten structures using the *Download all foldings* button.

(i) Find the corresponding target structures of the siRNAs in every potential folding.

(j) Select the most promising siRNA target sites by checking the following points:

   (i) Number of identical or similar target sites. The program will give various structures and corresponding energies (ΔG). Good target accessibility in different structure predictions increases the possibility of overall target accessibility.

   (ii) Number of bonds. High number of bonds (base pairs) within the target sequence indicates a strong secondary structure and less accessibility for an siRNA.

(k) Determine the ΔG value of the local RNA target structure for the selected siRNA target sites:

   (i) Find the corresponding siRNA target site and determine all bases within the target site that are involved in forming a secondary structure. Add also bases or base pairs which are part of the close neighborhood, for example bases that form loops, even if these are not part of the siRNA target site.

   (ii) Go to *Thermodynamic Details* of chosen structure in the Output window (folding results).

   (iii) Find the corresponding bases (marked by numbers) and add all single ΔG values of involved bases. Energies of helices and loops have to be included if one base of this structure is part of the target site. This calculation results in an approximation of ΔG_{local} which is usually sufficient. If precise calculation of ΔG_{local} is necessary, please refer to the Turner energy rules (Table 3).

(iv) Calculate the change in Gibbs free energy (ΔG) of all local RNA target structures (ΔG_{local}).

Exclude all siRNAs whose target structure showed strong variability based on at least ten folded structures ($\geq$5 different target structures). Furthermore, accessible target sites are characterized by low ΔG_{local} values (18). Therefore, select siRNAs in the order of their ΔG_{local} values with a threshold of about –15 kcal/mol (19). The selected siRNAs are potentially efficient siRNAs and their silencing efficiency can further be tested in Reporter Assays (see Subheading 3.2 and Note 6).

3.2. In Vitro Testing: Reporter Assays

Reporter assays are the method of choice to verify the efficiency of the selected siRNAs. The virus target sequence is cloned upstream or downstream of a GFP or luciferase reporter and is then cotransfected with the selected siRNAs. The virus target sequence, which is a partial viral sequence, should be long enough to simulate the structural environment of siRNA target sequence (20). In contrast, very long sequences may complicate reverse transcription and/or PCR amplification of the viral target sequence and may negatively influence the transfection efficiency of eukaryotic cells (see Note 7).

3.2.1. GFP Reporter Assay

The protocol for TOPO cloning described in the following section is a shortened version of the instruction manual (see Note 8). The TOPO cloning allows the ligation of the PCR-amplified target sequence of the virus into either pcDNA3.1/CT-GFP-TOPO or pcDNA3.1/NT-GFP-TOPO. The terms CT and NT indicate that GFP will be expressed fused to, respectively, the C-terminus and N-terminus of the target sequence.

1. Isolate the virus DNA or RNA using a commercial DNA or RNA virus isolation kit.

2. Perform a reverse transcription reaction if the isolated virus contains an RNA genome.

3. Design the primer for TOPO cloning. Do not add 5′ phosphates to the PCR primers. The pcDNA3.1/CT-GFP-TOPO does not contain an ATG initiation codon. The PCR product must, therefore, be cloned in frame with GFP. If the target sequence to be amplified lacks the ATG initiation codon, then the Kozak consensus sequence (Kozak consensus sequence is: (G or A)NNATGG (N = any base)) needs to be incorporated into the forward primer. The PCR product for pcDNA3.1/NT-GFP-TOPO may also be cloned in frame with GFP, but this is not essential if a translational stop codon is introduced upstream of the virus target sequence. We observed an improved GFP expression using this modified reporter

plasmid, in which the viral target sequence is part of the untranslated region of the GFP reporter. The stop codon can be incorporated into the forward primer, for example: 5′-**TGA** NNN N…

4. Run the PCR to amplify your target cDNA sequence.

5. Gel-purify the DNA fragment of interest (partial virus cDNA target sequence).

6. Add Taq DNA polymerase buffer, 0.4 mM dATP, and 0.5 U of Taq DNA polymerase to the gel-purified DNA fragment (see Note 9).

7. Incubate 10 min at 72°C.

8. Place the vials on ice.

9. Add 1 μl of salt solution and 1 μl of TOPO vector to 4 μl of the PCR product to set up the TOPO Cloning reaction (see Note 10).

10. Mix gently and incubate for 5 min at room temperature.

11. Place the tube on ice and store the sample at –20°C until later use, or proceed directly with transformation.

12. Thaw a tube containing 100 μl chemically competent *Escherichia coli* cells. Alternatively, use 50 μl TOP10 cells provided with the GFP Fusion TOPO TA Expression Kit (see Note 11).

13. Add 2 μl of the TOPO Cloning reaction (ligation reaction) to the tube containing 100 μl of chemically competent *E. coli* cells and mix gently. Do not mix by pipetting up and down.

14. Place the tube on ice and incubate for 30 min.

15. Heat-shock the *E. coli* cells for 60 s at 42°C without shaking.

16. Transfer the tube to ice and incubate for 3 min.

17. Add 700 μl of room temperature LB medium without antibiotics.

18. Cap the tube tightly and shake the tube horizontally at 37°C for 45 min.

19. Sediment the transformed *E. coli* cells by centrifugation at 3,000 × *g* for 5 min and discard 700 μl of the supernatant.

20. Resuspend the *E. coli* cells in residual LB medium.

21. Spread all of the transformation on a prewarmed selective plate (containing the antibiotic ampicillin) and incubate overnight at 37°C.

22. Pick ten colonies.

23. Culture all colonies overnight in 3 ml LB medium containing 100 μg/ml ampicillin.

24. Isolate plasmid DNA. In general, low amount and purity are sufficient for restriction analysis and/or sequencing.

25. To verify the orientation of the insert, analyze the plasmids by restriction analysis. To this end, it is advantageous to use at least one restriction site located within the virus target sequence and one that is present in the vector (see Note 12).

26. Clones containing inserts in the correct orientation should be sequenced to confirm that the original sequence has not been mutated (see Subheading 2.1, items 8 and 9).

27. Culture the clone of choice in 200 ml LB medium containing 100 μg/ml ampicillin.

28. Isolate the plasmid DNA. Plasmid DNA for transfection into eukaryotic cells must be very clean and free from phenol and sodium chloride to prevent cell death and to avoid low transfection efficiencies.

3.2.2. Dual-Luciferase Reporter Assay

The Dual-Luciferase Reporter Assay is a method to measure the activity of both firefly and Renilla luciferases in one sample. The virus target sequence is cloned in the 3′ untranslated region (3′-UTR) of the Renilla luciferase gene. In-frame cloning is not required as the translational stop codon of Renilla luciferase is present in psiCHECK-2 vector (cloning of the virus target sequence into this vector is subsequently described). The firefly luciferase, which is also expressed by the psiCHECK-2 vector, allows normalization of the Renilla luciferase activity.

1. Isolate the virus DNA or RNA using commercially available DNA or RNA virus isolation kit.

2. Perform a Reverse Transcription reaction if the isolated virus is based on RNA.

3. As in-frame cloning with Renilla luciferase gene is not required, the design of PCR primers follows common rules. Both primers contain one restriction site that is used for cloning into psiCHECK-2 vector (see Note 13). For example, use:

Forward primer: ccg CTC GAG nnn nnn nnn nnn nnn …

Reverse primer: ata gtt ta G CGG CCG C nnn nnn nnn nnn nnn …

The enzyme recognition sites (Xho I in the forward primer and Not I in the reverse primer) are indicated by upper case letters. The lower case letters show additional nucleotides for efficient restriction of the site located at the end of the PCR fragment. The bases which are part of the viral target sequence are indicated as nnn.

4. Run a PCR to amplify your target cDNA sequence.

5. Gel-purify the DNA fragment of interest (partial virus cDNA target sequence) and prepare an analytic gel to estimate the DNA concentration.

6. Add 5 U of Not I and Xho I (or other enzyme if other restriction sites were used) per μg DNA and complement the reaction with buffer and BSA.

7. Incubate for 20 h at 37°C.

8. Inactivate the restriction enzymes by incubation at 65°C for 20 min.

9. Place the tube on ice.

10. Ligate the DNA fragment into the psiCHECK-2 vector that was previously digested with Not I and Xho I using a standard protocol.

11. Transform the ligation reaction into *E. coli* cells and analyze colonies as described in Subheading 3.2.1, steps 12–28.

12. Clones containing inserts in the correct orientation should be sequenced (see Subheading 2.2, item 5).

3.3. Cellular Assays: Transfection of Mammalian Cells

For a first functional assay of the siRNAs in a reporter assay, it is advisable to use the cell line which will also be employed for virus assays. As an example, a protocol for transient transfection of HeLa cells in 24-well plates using Lipofectamine 2000 transfection reagent will be described (see Note 14). The transfection efficiency into other cell lines can be tested by transfection of a random siRNA that is labeled with Cy3 at the 5′ end of the siRNA sense strand (siRNA can be transfected with or without a reporter plasmid). We recommend the use of Cy3, as FITC is not distinguishable from GFP in fluorescence microscopy.

1. Seed 5×10^4 HeLa cells per well in a volume of 500 μl medium without antibiotics.

2. Incubate overnight at 37°C and 5% CO_2.

3. Dilute 0.8 μg of the reporter plasmid (pcDNA3.1/NT-GFP-TOPO, pcDNA3.1/CT-GFP-TOPO or psiCHECK-2 with inserted virus target sequence) and 10 nM of siRNA (designed antiviral siRNA or a negative control siRNA with no matches either to the viral or target organism genome) in Opti-MEM or serum-free medium to a total volume of 50 μl. The molar concentrations are related to the final volume of 600 μl.

4. Premix 2 μl of Lipofectamine 2000 transfection reagent with 48 μl Opti-MEM.

5. Incubate for 5 min at room temperature.

6. Combine Lipofectamine and DNA/siRNA mixture and incubate for at least 20 min.

7. Add the final mix (100 µl) to the cells. Do not remove the culture medium.

8. Incubate overnight at 37°C and 5% CO_2.

3.4. Analysis of siRNA Silencing Efficiency

If one of the TOPO vectors described above served as a reporter in cotransfection with siRNAs (see Subheading 3.3), the first analysis of siRNA silencing efficiency can be performed using fluorescence microscopy. This requires a filter for GFP detection. After replacement of medium from the transfected culture cells with 500 µl PBS, GFP expression of the reporter plasmid is determined (see Fig. 3). Highly efficient siRNAs strongly reduce GFP expression compared to the transfection of reporter plasmid without an siRNA, while the control siRNA should have no effect. These results should be confirmed by Western Blot analysis as described in Subheading 3.4.1. In the case of psiCHECK-2 cotransfection, both Renilla and Firefly luciferases will be measured (see Subheading 3.4.2).

3.4.1. Western Blot

1. Remove the medium from the culture cells.

2. Wash the cells with 500 µl of PBS.

3. Remove PBS by aspiration.

4. Add 30 µl of DTT lysis buffer per well and transfer the lysate to an eppendorf tube.

5. Boil the lysate for 5 min at 95°C.

Fig. 3. Fluorescence microscopy of GFP reporter assay. The viral sequence encoding the RNA-dependent RNA polymerase (3D^{Pol}) of Echovirus 30 (EV-30) was cloned downstream of GFP. Cells were transfected with 0.8 µg of GFP-3D^{Pol} plasmid and different siRNAs targeting 3D^{Pol} of EV-30. Cotransfection of 10 nM of siRNA2 to siRNA6 strongly reduced GFP expression compared to the transfection of reporter plasmid without an siRNA. The siRNA1 only leads to partial inhibition of target gene expression. In the panel labeled without siRNA, the cells were transfected with GFP reporter, but without siRNA. The control siRNA panel shows the results of a control siRNA with no matches either to the viral or target organism genome.

6. Separate the proteins on a 15% (w/v) SDS-polyacrylamide gel (see Note 15).

7. Transfer the proteins to PVDF membrane using your method of choice.

8. Control the protein transfer by Ponceau staining. Strip the membrane by washing with TBS.

    Shake the membrane during the following washing steps and antiserum incubations.

9. Rinse the membrane in TTBS.

10. Block with 5% non-fat milk powder in TTBS for 1 h at room temperature.

11. Rinse the membrane 3 times in TTBS.

12. Wash 3 times for 10 min in TTBS.

13. Incubate with GFP antiserum at a dilution of 1:5,000 in TTBS for 1 h at room temperature or overnight at 4°C.

14. Rinse the membrane 3 times in TTBS.

15. Wash 3 times for 10 min in TTBS.

16. Incubate with the secondary Goat Anti-Rabbit antibody conjugated with horseradish peroxidase, at a dilution of 1:5,000 in TTBS for 1 h at room temperature or overnight at 4°C.

17. Rinse the membrane 3 times in TTBS.

18. Wash 3 times for 10 min in TTBS.

19. Combine 500 μl of each component of the ECL Western Blotting Substrate and transfer the mixture to the membrane.

20. Incubate 1 min in the dark.

21. Detect the signals using your method of choice (Chemiluminescence Imager or development using an X-ray film).

22. Strip the membrane by washing 3 times for 10 min in TTBS.

23. Block with 5% nonfat milk powder in TTBS for 1 h or with 10% non-fat milk powder in TTBS for 10 min at room temperature.

24. To confirm equal loading of the samples, reprobe the membrane with an antibody directed against a housekeeping gene, for example, actin. Repeat all steps starting from step 9. Use the mouse monoclonal anti-Actin antibody at a dilution of 1:5,000.

3.4.2. Dual-Luciferase Measurement

1. Remove the growth medium from the culture cells.

2. Wash the cells with 500 μl of PBS.

3. Remove PBS by aspiration.

4. Add 100 µl of 1× passive lysis buffer (PLB) per well and shake the culture plate gently on an orbital shaker or on a rocking platform for 15 min at room temperature. The culture plate can be stored at –20°C for later use, or analyzed directly.

5. Transfer the lysate to a tube and sediment residual cell debris by centrifugation for 30 s at full speed in a tabletop centrifuge. Transfer the cleared lysates to a fresh tube for subsequently measurement.

6. Thaw adequate aliquots of LARII reagent (see Note 16) and prepare an adequate amount of Stop & Glo reagent (50 µl of each reagent per measurement is needed). As the Stop & Glo substrate is supplied in a 50× concentration, dilute 1 volume of 50× Stop & Glo substrate in 50 volumes Stop & Glo buffer. Protect both reagents from light and keep all components at room temperature during measurement.

7. Program your luminometer. (Default parameters: 2 s premeasurement delay and 10 s measurement period for each reporter.)

8. Pipette 50 µl of LARII reagent into a luminometer tube.

9. Add 20 µl of the sample (PLB lysate) or diluted sample and mix gently.

10. Measure the activity of Firefly luciferase.

11. Add 50 µl of Stop & Glo reagent and mix gently.

12. Measure the activity of Renilla luciferase.

13. Repeat the measurement for all samples starting from step 8.

Select efficient siRNAs for the following functional analysis in virus assay. Highly potent antiviral siRNAs reduce the GFP expression virtually to completion (Western Blot) or show at least 80% inhibition of Renilla luciferase (Dual-Luciferase Reporter Assay).

3.5. Virus Assays: Functional Analysis

A crucial test is the evaluation of the antiviral activity of designed siRNAs in assays with infectious virus. Two methods for in vitro testing are described below. The cell viability assay is an indirect method to quantify the efficiency of an siRNA, whereas the plaque reduction assay can show the direct inhibition of virus replication by an siRNA.

3.5.1. Cell Viability Assay

The XTT-based cell viability assay is used for the quantification of cell proliferation and viability. Metabolically active cells cleave the yellow tetrazolium salt to an orange formazan dye. The absorbance measured at a defined wavelength directly correlates with cell viability. We recommend the use of the XTT reagent due to its high sensitivity compared to other tetrazolium salts, such as MTT.

1. Seed $2–3 \times 10^4$ HeLa cells per well in a volume of 100 µl medium without antibiotics in a 96 well plate.

2. Incubate overnight at 37°C and 5% CO_2.
   Prepare the following siRNA transfection mixtures in quadruplicate.

3. Dilute siRNA (designed antiviral siRNA or a negative control siRNA with no matches either to the viral or target organism genome) in Opti-MEM or serum-free medium to a volume of 25 µl. The molar concentrations are related to the final volume of 150 µl.

4. Premix 0.5 µl of Lipofectamine 2000 transfection reagent with 24.5 µl Opti-MEM.

5. Incubate for 5 min at room temperature.

6. Combine Lipofectamine and siRNA and incubate for at least 20 min.

7. Add the final mix (50 µl per well) to the cells.

8. Incubate for 4 h at 37°C and 5% CO_2 (see Note 17).

9. Calculate the amount of virus required to reach a specific multiplicity of infection (m.o.i.) based on the titer of the virus stock and the number of seeded cells. Dilute the virus suspension to a specific m.o.i. An infection using an m.o.i. of 0.1 is subsequently described (see Note 18).
   Example: The virus titer is 2.5×10^7 plaque forming units (pfu)/ml. If 50 µl per well of the virus suspension is added to the cells, the titer will be 1.25×10^6 pfu per well. This value is further divided by the number of seeded cells resulting in an absolute m.o.i. value. Finally, the dilution factor is calculated by dividing by the desired m.o.i.

10. Prepare the dilution of virus suspension in refrigerator-cold serum-free medium without antibiotics (see Note 19).

11. Incubate at 37°C for 5 min before adding to the cells. Virus dilutions can be stored at 4°C for several minutes, if necessary.

12. Add 50 µl per well of the diluted virus suspension. Include negative control wells that remain uninfected. To this end, use serum-free medium without antibiotics and handle these cells identical to infected cells.

13. Incubate for 30 min at 37°C and 5% CO_2 (see Note 20).

14. Remove the virus suspension and add 100 µl of medium containing FCS and antibiotics.

15. Incubate at 37°C and 5% CO_2 until beginning the cell viability assay (XTT measurement).

16. Prepare an adequate amount of XTT reagent. For example, mix 100 μl electron-coupling reagent with 5 ml XTT labeling reagent.

17. Add 50 μl per well of fresh XTT reagent and incubate 4 h at 37°C and 5% CO_2. Do not remove the medium.

18. Measure the absorbance in a microtiter plate reader at a defined wavelength between 450 and 500 nm. The reference wavelength should be greater than 650 nm.

3.5.2. Plaque Reduction Assay

Generally, each plaque has its origin in the viral infection of a single cell. The reduction of virus titer shows directly the efficiency of an siRNA as the virus spread is prevented.

1. Seed $1.5–2.5 \times 10^5$ HeLa cells per well in a volume of 500 μl of medium without antibiotics in a 24 well plate.

2. Incubate overnight at 37°C and 5% CO_2.

3. Dilute siRNA (designed antiviral siRNA or a negative control siRNA with no matches either to the viral or target organism genome) in Opti-MEM or serum-free medium to a volume of 50 μl. The molar concentrations are related to the final volume of 600 μl.

    Prepare as many siRNA transfected wells as needed to titrate the different virus dilutions. Include positive control wells which remain untransfected, but will be infected with different virus dilutions.

4. Premix 2 μl of Lipofectamine 2000 transfection reagent with 48 μl Opti-MEM.

5. Incubated for 5 min at room temperature.

6. Combine Lipofectamine and siRNA and incubate for at least 20 min.

7. Add the final mix (100 μl per well) to the cells.

8. Incubate for at least 4 h at 37°C and 5% CO_2 (see Note 17).
    Cells should be close to 100% confluent before overlaying the infected cell monolayer.

9. Prepare serial 1:10 dilutions of the virus stock in refrigerator-cold serum-free medium without antibiotics (see Note 19). In general, an appropriate range of dilutions is 10^{-4} to 10^{-7} (depending on the titer of virus stock).

10. Incubate at 37°C for 5 min before adding to the cells. Virus dilutions can be stored at 4°C for several minutes, if necessary.

11. Add 250 μl per well of the diluted virus suspension. Include negative control wells that remain uninfected. To this end, use serum-free medium without antibiotics and handle these cells identically to infected cells.

12. Incubate for 30 min at 37°C and 5% CO_2 (see Note 20).

13. Remove the virus suspension by gentle aspiration.

14. Add 4 ml FCS to 20 ml Eagle-MEM and prewarm to 41.5°C.

15. Melt 0.28 g of Difco Agar Noble in 16 ml H_2O. Cool to 40–45°C and add the prewarmed Eagle-MEM to the Agar Noble solution.

16. Incubate for 15 min at 41.5°C. This makes a 0.7% Agar Noble solution (Eagle Overlay) which is used to overlay the infected cell monolayer.

17. Add 500 µl per well of Eagle Overlay and leave the plates for 5 min at room temperature. Take care not to dislodge any cells.

18. Incubate at 37°C and 5% CO_2. Plaques should be visible within around 3 days depending on the virus.

19. Add 500 µl of neutral red solution to each of the wells and incubate for 3–4 h at 37°C and 5% CO_2 (see Note 21).

20. Remove the stain by gentle aspiration.

21. Invert the plate and count the plaques in each well.

22. Calculate the viral titer of each well.

$$\text{pfu/ml} = \text{counted plaques} / (\text{dilution factor} \times \text{volume of diluted virus added to the well}).$$

An alternative protocol can be used to accelerate the staining (see Note 22). Efficient siRNAs are able to reduce the virus titer at least one order of magnitude. Figure 4 shows an example of such a plaque reduction assay.

3.6. Chemical Modification: Generating Stabilized siRNAs

Selected (antiviral) siRNAs which show high efficiency may be chemically modified to decrease potential off-target effects and/or to enhance serum stability. One approach to reduce off-target effects is to prevent the incorporation of the unwanted sense strand in RISC. This can, for example, be achieved by an LNA modification at the 5′ end (21), or by introduction of a 2′-O-methyl modification at position 1 and 2 (13) of the sense strand. However, a specific reduction of off-target effects was observed when position 2 of the antisense strand was additional 2′-O-methyl modified (13). In theory, chemical modifications of an siRNA will also result in increased serum stability. In practice, siRNA modifications are often accompanied by decreased silencing efficiency compared to the unmodified siRNA. LNA modifications introduced at any position except 1, 10, 12, and 14 in the antisense strand do not cause substantial loss of silencing efficiency (21). Another way to combine high silencing efficiency, increased serum stability, and reduced off-target effects is complete modification
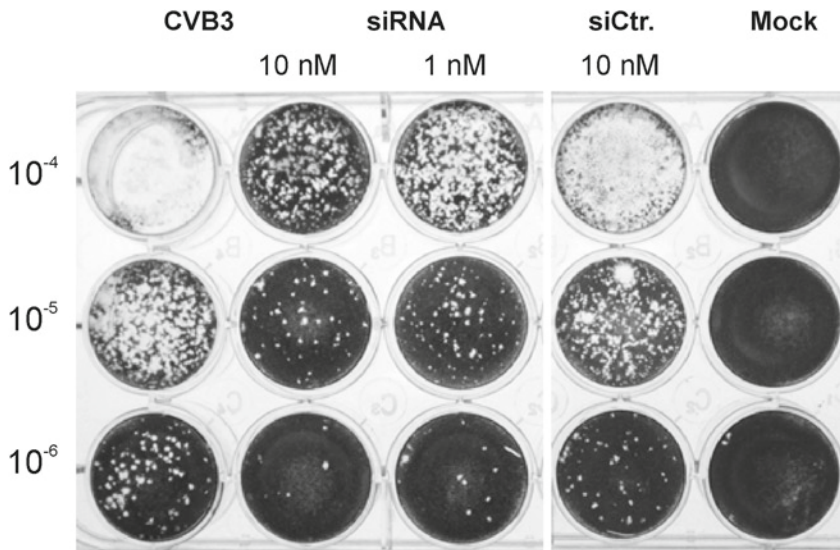
Fig. 4. Plaque reduction assay. After transfection of a virus-specific siRNA at 1 and 10 nM concentrations, cells were infected with different dilutions of Coxsackievirus B3 (CVB3). The infection with a virus dilution of 10^{-5} of 10 nM transfected cells targeting CVB3 resulted in fewer visible plaques than for the 10^{-6} dilution of the virus alone. The CVB3 titer was reduced more than one order of magnitude. Mock: uninfected cells, siCtr.: control siRNA with no matches to either the viral or target organism genomes.

with different nucleotide analogs as shown for an siRNA targeting the HBV genome (12) (Fig. 5). However, chemical modifications are dependent on the application as well as on the virus to be inhibited.

With the strategy described here, it will be possible to identify efficient siRNAs against virtually any given virus – as we have recently exemplified for echovirus 30 (22). The next step will be to transfer the antiviral RNAi approach to an in vivo model. One of the main challenges will then be efficient delivery of the siRNA to the desired target tissue. The following chapters describe virus-based approaches to deliver shRNA expression cassettes as well as different strategies for the application of chemically presynthesized siRNAs.

4. Notes

1. In order to identify conserved regions in the viral genome, it is advisable to select sequences of various strains and perform a BLAST search. Regions with high homology can be considered conserved and represent suitable siRNA target sites.

2. Tuschl's motif pattern strongly restricts the number of siRNAs for subsequent selection, which may become a problem if short sequences are targeted.

sense   5'   B **GGA**cuucucuc**AA**uuuucu**TT** B 3'
                         ┊┊┊┊┊┊┊┊┊┊┊┊┊┊┊┊┊┊┊
antisense   3'   **T**ₛ**T**ccuGAAGAGAGuuAAAAGA   5'

AGT = deoxy A, G &T
AG   = 2'-O-methyl A & G
cu   = 2'-fluoro C & U
B   = 3',5' inverted deoxy abasic
s   = phosphorothioate

    2'-O-methyl   2'-fluoro   phosphorothioate   3',5' inverted
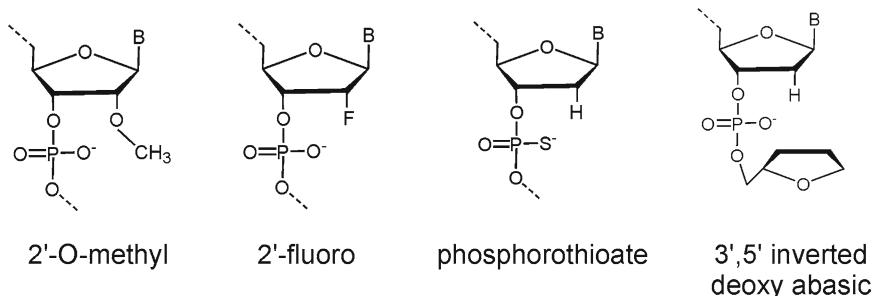   deoxy abasic

Fig. 5. Chemical modifications of the hepatitis B virus siRNA (figure modified from ref. (12)). The siRNA is fully modified (*top*) with different nucleotide analogs (*bottom*) and showed an increased half-life in human serum and a significantly higher activity in a vector-based in vivo model of HBV infection as compared to the unmodified form.

3. The NCBI BLAST tool or/and the Ensembl BLAST tool can be used for homology searches (see Table 3).

4. If the summary of potential folding is: "No folding possible" and/or the software are/is showing the term ∞ for ΔG, then the RNA sequence does not form any secondary structure.

5. If all siRNAs (antisense strand) give negative ΔG values, examine all structures again and use only siRNAs that show stem-loop structures with ≥2 nucleotide free at the 5′ terminus and ≥4 free nucleotides at the 3′ terminus for further analyses (17).

6. If no siRNAs meet the indicated criteria, first check if another partial sequence of the virus can be targeted. Alternatively, the threshold for ΔG of the local RNA target site can be adjusted, especially if many siRNAs remain whose antisense strands do not form stable secondary structures (see also Note 5). To this end, select at least four siRNAs in order of their ΔG_{local} values. The threshold of Reynolds' criteria should not be changed.

7. In general, it is best to include a partial target RNA with a length of 1–2 kb that spans the siRNA target sequence.

8. Both an extended protocol and a trouble shouting guide of TOPO cloning can be found in the complete instruction manual provided with the kit.

9. For long virus target sequences, use a polymerase with proof-reading activity. Since the amplified sequences will lack 3′ A-overhangs, which are essential for the described cloning method, additional incubation with *Taq* polymerase is necessary.

10. If *E. coli* is to be transformed by electroporation, the salt solution must be diluted fourfold to a final concentration of 50 mM NaCl and 2.5 mM $MgCl_2$ to prevent arcing.

11. Optionally, 1.5 μl of 1.42 mM β-mercaptoethanol can be added to the chemically competent *E. coli* cells followed by an incubation on ice for 20 min. This procedure may increase the transformation efficiency. Store the 1.42 mM solution of β-mercaptoethanol at 4°C.

12. Vector maps and sequences of pcDNA3.1/CT-GFP-TOPO and pcDNA3.1/NT-GFP-TOPO are available on the Invitrogen website (see Table 3). Vector map and sequence of psiCHECK-2 is available on the Promega website (see Table 3).

13. Other restriction sites may also be suitable. In this case, customize the additional bases for efficient restriction and the incubation time. An extensive table is provided by New England Biolabs (see Table 3).

14. The use of other cell lines or/and other transfection reagents will require different conditions than those given here.

15. Density of SDS-polyacrylamide gel depends on molecular weight (MW) of the target sequence. If pcDNA3.1/CT-GFP-TOPO was used, add the MW of GFP (around 15 kD) to the calculated MW of the target sequence for an estimate, though some sequences may influence gel mobility, leading to measured weights other than the predicted value.

16. LARII aliquots have to be thawed at room temperature, as the reagent is heat-labile.

17. Incubation overnight is also possible if the cells are plated at a lower density.

18. An m.o.i. of 0.1 is suitable for many viruses, but in some cases may need to be adjusted. The control infection should show as many individual plaques as can be reliably counted. An effective siRNA will appreciably reduce the number of plaques, while a weak siRNA may only lead to a slight reduction. A large number of distinguishable plaques on the control plate will simplify the recognition of weak siRNAs, which may be desirable if further optimization is possible.

19. FCS within a medium may interfere with viral infection efficiency.

20. Incubation time depends on the infection cycle of a specific virus.

21. Neutral red as well as crystal violet is taken up by healthy cells, but not by dead cells. Plaques therefore appear as clear circles against the red/violet background. If the clear areas overlap too much, it is difficult to recognize individual plaques and infection time should be reduced. If plaques are too small to be easily read, try extending infection time, though it could also be an indication of a defective or weakened strain.

22. The staining can be accelerated by using crystal violet solution. To this end, fix the cells with 10% TCA for 10 min at room temperature. Then remove the agar without disturbing the cell monolayer and add 500 µl of crystal violet solution (0.5% solution in PBS) to each of the wells and incubate for 5 min at room temperature (see Note 21). Remove the stain by aspiration and wash with PBS. Invert the plate, and count the plaques of each well. Calculate the viral titer as described in Subheading 3.5.2, step 22.

References

1. Fox, J. L. (2007) Antivirals become a broader enterprise. *Nat Biotechnol* **25**, 1395–1402.

2. Grimm, D. (2009) Small silencing RNAs: state-of-the-art. *Adv Drug Deliv Rev* **61**, 672–703.

3. Kurreck, J. (2009) RNA interference: from basic research to therapeutic applications. *Angew Chem Int Ed Engl* **48**, 1378–1398.

4. Castanotto, D., and Rossi, J. J. (2009) The promises and pitfalls of RNA-interference-based therapeutics. *Nature* **457**, 426–433.

5. Haasnoot, J., Westerhout, E. M., and Berkhout, B. (2007) RNA interference against viruses: strike and counterstrike. *Nat Biotechnol* **25**, 1435–1443.

6. Kurreck, J. (2006) siRNA efficiency: structure or sequence-that is the question. *J Biomed Biotechnol* **2006**, 83757.

7. Wang, X., Wang, X., Varma, R. K., Beauchamp, L., Magdaleno, S., and Sendera, T. J. (2009) Selection of hyperfunctional siRNAs with improved potency and specificity. *Nucleic Acids Res* **37**, e152.

8. Lee, H. S., Ahn, J., Jee, Y., Seo, I. S., Jeon, E. J., Jeon, E. S., Joo, C. H., Kim, Y. K., and Lee, H. (2007) Universal and mutation-resistant anti-enteroviral activity: potency of small interfering RNA complementary to the conserved cis-acting replication element within the enterovirus coding region. *J Gen Virol* **88**, 2003–2012.

9. ter Brake, O., t Hooft, K., Liu, Y. P., Centlivre, M., von Eije, K. J., and Berkhout, B. (2008) Lentiviral vector design for multiple shRNA expression and durable HIV-1 inhibition. *Mol Ther* **16**, 557–564.

10. Merl, S., and Wessely, R. (2007) Anti-coxsackieviral efficacy of RNA interference is highly dependent on genomic target selection and emergence of escape mutants. *Oligonucleotides* **17**, 44–53.

11. Behlke, M. A. (2008) Chemical modification of siRNAs for in vivo use. *Oligonucleotides* **18**, 305–319.

12. Morrissey, D. V., Blanchard, K., Shaw, L., Jensen, K., Lockridge, J. A., Dickinson, B., McSwiggen, J. A., Vargeese, C., Bowman, K., Shaffer, C. S., Polisky, B. A., and Zinnen, S. (2005) Activity of stabilized short interfering RNA in a mouse model of hepatitis B virus replication. *Hepatology* **41**, 1349–1356.

13. Jackson, A. L., Burchard, J., Leake, D., Reynolds, A., Schelter, J., Guo, J., Johnson, J. M., Lim, L., Karpilow, J., Nichols, K., Marshall, W., Khvorova, A., and Linsley, P. S. (2006) Position-specific chemical modification of siRNAs reduces "off-target" transcript silencing. *RNA* **12**, 1197–1205.

14. Dutkiewicz, M., Grunert, H. P., Zeichhardt, H., Lena, S. W., Wengel, J., and Kurreck, J. (2008) Design of LNA-modified siRNAs against the highly structured 5′ UTR of coxsackievirus B3. *FEBS Lett* **582**, 3061–3066.

15. Reynolds, A., Leake, D., Boese, Q., Scaringe, S., Marshall, W. S., and Khvorova, A. (2004) Rational siRNA design for RNA interference. *Nat Biotechnol* **22**, 326–330.

16. Zuker, M. (2003) Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res* **31**, 3406–3415.

17. Patzel, V., Rutz, S., Dietrich, I., Koberle, C., Scheffold, A., and Kaufmann, S. H. (2005) Design of siRNAs producing unstructured guide-RNAs results in improved RNA interference efficiency. *Nat Biotechnol* **23**, 1440–1444.

18. Schubert, S., Grunweller, A., Erdmann, V. A., and Kurreck, J. (2005) Local RNA target structure influences siRNA efficacy: systematic analysis of intentionally designed binding regions. *J Mol Biol* **348**, 883–893.

19. Westerhout, E. M., Ooms, M., Vink, M., Das, A. T., and Berkhout, B. (2005) HIV-1 can escape from RNA interference by evolving an alternative structure in its RNA genome. *Nucleic Acids Res* **33**, 796–804.

20. Sun, G., and Rossi, J. J. (2009) Problems associated with reporter assays in RNAi studies. *RNA Biol* **6**, 406–411.

21. Elmen, J., Thonberg, H., Ljungberg, K., Frieden, M., Westergaard, M., Xu, Y., Wahren, B., Liang, Z., Orum, H., Koch, T., and Wahlestedt, C. (2005) Locked nucleic acid (LNA) mediated improvements in siRNA stability and functionality. *Nucleic Acids Res* **33**, 439–447.

22. Rothe, D., Werk, D., Niedrig, S., Horbelt, D., Grunert, H. P., Zeichhardt, H., Erdmann, V. A., and Kurreck, J. (2009) Antiviral activity of highly potent siRNAs against echovirus 30 and its receptor. *J Virol Methods* **157**, 211–218.