# Production of highly potent recombinant siRNAs in *Escherichia coli*

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Published online 31 October 2013; doi:10.1038/nprot.2013.149

**We recently invented a method to produce highly potent siRNAs in *Escherichia coli*, based on the serendipitous discovery that ectopic expression of p19, a plant viral siRNA-binding protein, stabilizes otherwise unstable bacterial siRNAs, which we named pro-siRNAs for prokaryotic siRNAs. We present a detailed protocol describing how to produce pro-siRNAs for efficiently knocking down any gene, beginning with the design of a pro-siRNA expression plasmid and ending with siRNA purification. This protocol uses one plasmid to co-express a recombinant His-tagged p19 protein and a long hairpin RNA containing sense and antisense sequences of the target gene. pro-siRNAs are isolated and purified using nickel beads and HPLC, using methods used to produce recombinant proteins. Once a pro-siRNA plasmid is obtained, production of purified pro-siRNAs takes a few days. The pro-siRNA technique provides a reliable and renewable source of siRNAs, and it can be implemented in any laboratory whose members are skilled in routine molecular biology techniques.**



## INTRODUCTION

RNA interference (RNAi), a ubiquitous phenomenon in which short, double-stranded (ds) RNAs called small interfering RNAs (siRNA) downregulate the expression of genes bearing complementary sequences, was first discovered 15 years ago in *Caenorhabditis elegans*[1] and shortly thereafter in plants[2]. siRNAs are predominately 21–24-nt long and are produced by the RNase III family protein Dicer from longer dsRNA precursors[3]. One strand of the siRNA duplex binds to Argonaute (Ago) proteins, a family of RNase H–like proteins, which can then slice or repress translation of an mRNA on the basis of sequence complementarity[4,5]. In eukaryotes, endogenous siRNA pathways have many roles, including repressing repetitive and transposable genomic elements[6] and defending the host against infection by RNA viruses[7].

Transfection of synthetic siRNAs that mimic endogenous siRNAs can take advantage of the ubiquitous RNAi machinery to knock down gene expression in mammalian cells[8,9]. RNAi has greatly facilitated the study of mammalian gene function and has shown great potential for therapeutic knockdown of disease-causing genes. Currently, the dominant method of gene knockdown uses chemically synthesized siRNAs. Synthetic siRNAs, like endogenous siRNAs, are typically ~21-nt long with 2-nt overhangs at the 3′ ends and phosphate groups at the 5′ ends. After siRNA transfection, target gene mRNA and protein knockdown occurs within 1–3 d. In addition to chemical synthesis, siRNAs can also be made by using RNase III family proteins, including bacterial RNase III[10] or eukaryotic Dicer[11], to digest T7 RNA polymerase–transcribed dsRNAs into siRNAs *in vitro*. Commercially available esiRNAs (for endoribonuclease-prepared siRNAs)[10], made by *Escherichia coli* RNase III *in vitro*, are highly efficient and are associated with few off-target effects[12,13]. Alternatively, plasmid or viral vectors can be used to deliver short hairpin RNA (shRNA)–expressing constructs[14], whose transcripts are processed by Dicer into functional siRNAs within the cell. When shRNA vectors are integrated into genomic DNA, gene knockdown lasts longer than transiently transfected synthetic siRNAs.

The use of bacteria to generate siRNAs can potentially reduce the cost of siRNAs and can provide a means of delivering siRNAs into cells. *E. coli* cells engineered to express a bacterial invasin have been used to deliver shRNAs into mammalian cells both *in vitro*[15,16] and *in vivo* in the gut[17]. Large quantities of long dsRNAs can be made in RNase III–deficient *E. coli* overexpressing T7 RNA polymerase[18] or φ6 RNA–dependent RNA polymerase[19]. Recently, we developed a protocol for producing highly potent and pure siRNAs directly from *E. coli* cells[20]. Those siRNAs, which induce ~90% gene knockdown when used at 2 nM concentrations[20], are generated from long dsRNAs (>100 nt in length) and contain multiple siRNA sequences. After HPLC purification, they do not stimulate innate immunity and have few off-target effects. By using this protocol, bacteria produce 'recombinant' and renewable[21] siRNAs in a process that resembles recombinant protein production.

### Development of the protocol

The protocol described here arose from our unexpected discovery that the expression of *p19*, a plant *Tombusvirus* RNAi suppressor gene[22], in *E. coli* cells, stabilizes siRNA-like small RNAs with similar chemical properties as eukaryotic siRNAs. p19 selectively binds to ~21-nt-long siRNAs without sequence specificity[23–25]. Endogenous siRNAs are made in bacteria from dsRNA precursors formed by pairing endogenous overlapping sense and antisense transcripts (L.H. and J.L., unpublished data). *E. coli* RNase III, which is capable of making ~21-nt-long siRNAs *in vitro* (under specific conditions)[10,26], is responsible for making siRNAs *in vivo*. Those siRNAs are generally not stable in wild-type bacteria, but they can be stabilized by ectopic expression of the siRNA-binding protein p19. We named siRNAs of bacterial origin pro-siRNAs for 'prokaryotic siRNAs'[20].

To produce pro-siRNAs against a gene target in *E. coli* cells, we engineered an *E. coli* plasmid to overexpress His-tagged *p19* (from a *tac* promoter) together with a long hairpin RNA containing the target sequence (expressed from a T7 promoter). Expression

of p19 and the hairpin-containing transcript are both induced by IPTG. Because overexpressed exogenous dsRNAs are much more abundant than endogenous dsRNAs, the resulting pro-siRNAs mainly comprise the target sequences (>82% by deep sequencing). Although pro-siRNAs contain some plasmid and bacterial endogenous sequences, they do not cause off-target effects when transfected into human cell lines, probably because of the low abundance of individual bacterial and plasmid sequences and their poor homology to mammalian genes[20]. pro-siRNAs are isolated from bacterial lysates using nickel (Ni) beads to capture His-tagged p19, and p19-bound pro-siRNAs are then selectively eluted with 0.5% (wt/vol) SDS. To remove bacterial endotoxin and longer contaminating RNAs, pro-siRNAs are further purified by anion-exchange HPLC. The final, purified pro-siRNA is a pool of many siRNA sequences across the target sequence, but the sequence distribution is uneven and contains some 'hot spot' sequences. When transfected at low-nanomolar concentrations in mammalian cells, pro-siRNAs knock down ~90% of the target gene expression and are as efficient as commercial synthetic siRNAs, tested in multiple human cell lines using *EGFP* and a handful of human and viral genes as targets[20].

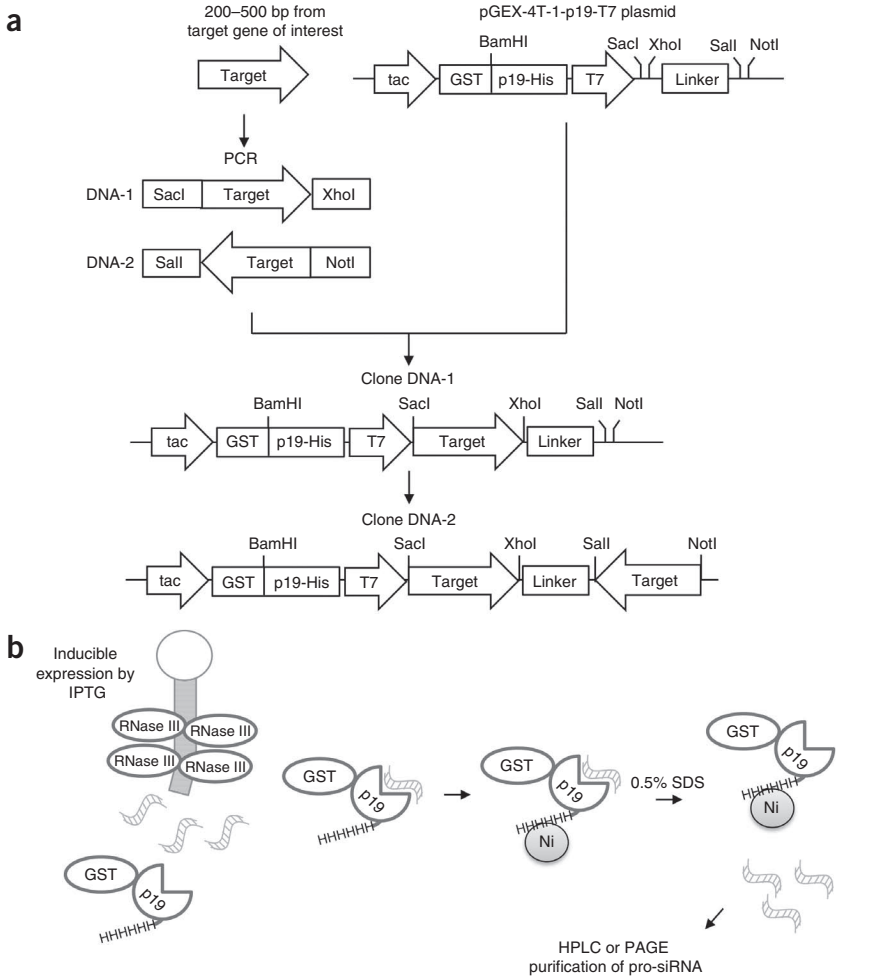


**Figure 1** | Workflow of the protocol to produce pro-siRNAs from *E. coli*. (**a**) pro-siRNA plasmid cloning procedure. (**b**) Expression and isolation of pro-siRNA.

This protocol uses a widely used glutathione S-transferase (GST) fusion protein–expressing system (pGEX-4T-1 plasmid)[27] to express a GST-p19-His fusion protein. The plasmid was further engineered to contain two pairs of restriction sites flanked by a linker sequence for cloning of a hairpin RNA construct (pGEX-4T-1-p19-T7). It is important to note that a relatively short hairpin RNA (100 bp) contained more *E. coli* endogenous sequences and had more off-target effects[20]. Therefore, we suggest that users design hairpins of at least 200 bp of target gene sequence.

### Experimental workflow of pro-siRNA production

A schematic of the protocol is shown in **Figure 1**. This protocol begins with the selection of a target sequence and cloning of a hairpin construct into the pGEX-4T-1-p19-T7 plasmid. We suggest generally selecting the first 200–500-bp sequence immediately after the ATG start site of the target gene. Users may also explore the use of a target dsRNA–selecting tool designed for producing esiRNAs (http://deqor.mpi-cbg.de)[11,28,29]. To make the hairpin plasmid, a two-step cloning process is involved: first, one copy of the target DNA is inserted into a set of two restriction sites (SacI and XhoI), and then the second copy of the target DNA is inserted in reverse orientation into another set of two restriction sites (SalI and NotI) (**Fig. 1a**). The four unique restriction sites and cloning primers are designed to ensure that two copies of the target sequence (referred to as DNA-1 and DNA-2, respectively) are in opposite orientation, so that they will form a hairpin.

The resulting pro-siRNA plasmid is then transformed into an *E. coli* host strain expressing T7 RNA polymerase (**Fig. 1b**). Bacterial culture conditions and subsequent pro-siRNA purification procedures are very similar to those used for expressing recombinant proteins. *E. coli* cells are cultured to exponential phase ($OD_{600}$ ~0.3–0.6), and IPTG is added to induce the expression of both GST-p19-His fusion protein and hairpin RNAs. After IPTG induction, *E. coli* cells are collected and lysed, and GST-p19-His proteins and associated pro-siRNAs are purified by Ni beads. pro-siRNAs, but not p19, are then eluted with 0.5% (wt/vol) SDS. pro-siRNAs of ~21 nt in length are further purified from longer RNAs by anion-exchange HPLC. After isopropanol precipitation, pro-siRNAs are ready for use. Because HPLC is not readily available in many laboratories, we present an alternative pro-siRNA purification method using native PAGE in **Box 1**. However, users should note that we have not tested whether endotoxin is removed from siRNAs purified by the PAGE method.

## Box 1 | pro-siRNA purification by native PAGE ● TIMING 1 d

▲ CRITICAL We have not tested whether endotoxin is removed from siRNAs purified by PAGE. Endotoxin contamination can be removed using commercially available kits, such as the Detoxi-Gel endotoxin removing gel (see also TROUBLESHOOTING).

1. Add a one-tenth volume of eluate (100 µl for 1 ml of eluate from Step 37 of the main PROCEDURE, either freshly prepared or stored at –20 °C) of 2 M KCl to SDS eluate from Step 37. Place the sample on ice for 10 min to precipitate SDS. Centrifuge the samples for 5 min at 16,000*g* at 4 °C.
2. Transfer the supernatant into two 1.5-ml microcentrifuge tubes, ~500 µl each. Add 50 µl of 3 M sodium acetate (pH 5.0) to the sample and mix. Thereafter, add one volume (550 µl) of isopropanol and prompt the precipitation of RNAs by incubating at –20 °C for over 1 h.
3. Centrifuge the samples for 30 min at 16,000*g* at 4 °C. Carefully discard most of the supernatant by aspiration or pipetting.
4. Add 1 ml of 75% (vol/vol) ethanol and invert the tubes several times to wash the pellets.

? TROUBLESHOOTING

5. Centrifuge the samples for 5 min at 16,000*g* at 4 °C. Carefully discard most of the supernatant by aspiration or pipetting.
6. Centrifuge the samples for another 2 min at 16,000*g* at 4 °C, and remove any remaining liquid using a 200-µl pipette tip.
7. Open the caps of the tubes to air-dry the pellet for 5 min.
8. Dissolve the pellets in a total of 100 µl of nuclease-free water.
9. Measure the concentration of RNAs using a NanoDrop or UV spectrophotometer.
10. Make a 15% (wt/vol) TBE polyacrylamide gel (see Reagent Setup). Mix the sample from Step 8 with 1 volume of gel loading buffer II (or other native RNA loading buffer) and load 500 ng of RNA into each well. Load 5 µl of siRNA marker into the first well.

▲ CRITICAL STEP Do not heat the samples after adding gel loading buffer to keep the RNAs in their native state.

11. Run the gel in 1× TBE buffer at 150 V until bromophenol blue is about 1 cm from the bottom.
12. Stain the gel with SYBR Gold in 1× TBE buffer for 5 min on an orbital shaker at low speed.
13. Use a UV or blue-light box to visualize the RNAs. Cut out a strip of gel containing RNA fragments in the 17–25-nt range, according to the markers. This strip of gel contains the desired ~21-nt pro-siRNAs.

? TROUBLESHOOTING

14. Cut the gel strip into small pieces (~2 mm × 2 mm), and carefully transfer them into a 1.5-ml microcentrifuge tube (or multiple tubes if needed).
15. Elute pro-siRNAs by adding 500 µl (or any volume that can at least cover the gel) of 0.3 M NaCl, and rotate the tube overnight at 4 °C.
16. Pass the eluate through a 0.22-µm centrifuge tube filter.
17. Precipitate pro-siRNAs by adding 500 µl of isopropanol (for every 500 µl eluate) and by incubating at –20 °C for over 1 h. Ten micrograms of glycogen can be added as a carrier for precipitation.
18. Repeat Steps 3–8. Measure the final concentration of the pro-siRNA product by NanoDrop or UV spectrophotometer. An example of PAGE-purified pro-siRNA is shown in **Figure 3b**. Test endotoxin contamination using the LAL gel clot assay (Pyrogent).

? TROUBLESHOOTING



Endotoxin contamination can be tested and removed with commercially available kits.

### Advantages and limitations of pro-siRNA

pro-siRNAs are produced using reagents and equipment that are commonly found in molecular biology laboratories. The protocol presented herein enables any laboratory to produce homemade highly potent siRNAs on demand, once a target sequence is determined and the pro-siRNA plasmid is constructed. pro-siRNAs are easy to design and provide a renewable and reliable source of potent siRNAs that offer highly reproducible results and low off-target effects. The production of pro-siRNAs can be scaled up easily, so producing large quantities of siRNAs, such as for many *in vitro* experiments or for animal experiments, could be very cost-effective. Moreover, all the hairpins designed against multiple genes using this protocol resulted in at least 90% gene knockdown after transfection of 2 nM pro-siRNA. Therefore, the cost, labor and time lost in the trial-and-error approach of trying multiple chemically synthesized siRNAs to obtain a single effective sequence can be avoided.

Because a pro-siRNA is a pool of many different siRNA sequences targeting the same gene, a single pro-siRNA preparation directed against HIV-1 *gag* was able to target multiple strains of HIV-1 virus, which had only ~75% sequence identity[20,30]. Thus, pro-siRNAs could be especially suitable for targeting viral genes that are heterogeneous in different subtypes or can mutate, or for genes with a lot of sequence variation (such as major histocompatibility genes or genes that correspond to polymorphic receptors). In principle, this approach could also be used to knock down families of homologous genes or to produce siRNAs that cross-react with the orthologous gene from multiple species with a single construct. Depending on sequence conservation, the method might be used to knock down all the genes that contain a shared homologous functional exonic motif. In contrast, caution is advised when one is planning to target an individual member of a closely related multigene family. In that case, instead of targeting the coding region, more variable sequences, such as in the 3′ UTR, should be chosen as target sequences for the hairpins.

Most laboratories are not capable of chemical synthesis of siRNAs, but synthetic siRNAs are easy to order from many

vendors. However, because current computational algorithms for predicting effective siRNA sequences are far from perfect[31], making and testing multiple siRNA sequences to identify one potent sequence can be costly and time-consuming, if there are not already validated siRNAs for the target gene of interest. However, chemical synthesis offers many types of modifications that can improve stability or efficiency of siRNAs and/or reduce immunogenicity[32,33]. It is not clear what modifications could be incorporated, or how efficiently, during bacterial production by feeding bacteria with altered nucleotides or by modifying purified pro-siRNAs.

Making siRNAs enzymatically requires enzymes and chemicals of high quality, which are expensive, and the process is laborious. In contrast, the production of pro-siRNA, being a cell-based approach, requires only simple bacterial culture medium and commonly used reagents. Thus, it may be easier and more economical when the protocol is fully optimized.

However, some potential drawbacks of the pro-siRNA technology exist, which might be alleviated in the future. To implement the protocol, about a week's worth of work is necessary to construct the pro-siRNA plasmid. In the future, commercial vendors might supply ready-made pro-siRNA plasmids for at least some commonly studied mammalian genes. The current yield of pro-siRNAs is low (about 40 µg per liter of culture), but the plasmid, *E. coli* strain and/or the production protocol could be optimized to improve the yield. As more people adopt and improve this protocol, the pro-siRNA technology could become more user-friendly and more efficient.



## MATERIALS

### REAGENTS

- Tris-HCl, 1 M, pH 7.0 (Life Technologies/Ambion, cat. no. AM9851)
- SDS, 10% (wt/vol) (Fisher Scientific, cat. no. BP2430-1) **! CAUTION** SDS is corrosive. Wear a lab coat and protective gloves when handling it.
- TBE, 10× (Life Technologies/Ambion, cat. no. AM9863)
- Acrylamide and bis-acrylamide solution, 40% (wt/vol), 19:1 (Bio-Rad, cat. no. 161-0144) **! CAUTION** Acrylamide is highly toxic. Avoid spilling and contact with skin. Wear a lab coat and protective gloves when handling it.
- Agarose (Denville, cat. no. CA3510-8)
- Ammonium persulfate (Sigma-Aldrich, cat. no. A3678) **! CAUTION** Ammonium persulfate is toxic and reactive. Wear a lab coat and protective gloves when handling it.
- Ampicillin sodium salt (Sigma-Aldrich, cat. no. A0166)
- Carbenicillin disodium salt (Sigma-Aldrich, cat. no. C3416)
- Detoxi-Gel endotoxin removing gel (Thermo Fisher Scientific, cat. no. 20339)
- DNA template (see Reagent Setup)
- Ethanol (VWR/KOPTEC, cat. no. V1016) **! CAUTION** Ethanol is flammable. Keep it away from fire.
- Ethidium bromide (Sigma-Aldrich, cat. no. E7637) **! CAUTION** Ethidium bromide is toxic. Wear a lab coat and protective gloves when handling it.
- EDTA (Sigma-Aldrich, cat. no. EDS)
- Gel loading buffer II (Life Technologies/Ambion, cat. no. AM8546G)
- Glacial acetic acid (Fisher Scientific, cat. no. BP2401-212)
- Glycogen (Life Technologies/Invitrogen, cat. no. 10814-010)
- HisPur Ni-NTA resin (Thermo Fisher Scientific, cat. no. 88222)
- HPLC-grade water (Sigma-Aldrich, cat. no. 270733)
- Imidazole (Sigma-Aldrich, cat. no. I5513)
- IPTG (Gold Biotechnology, cat. no. I2481C100)
- Isopropanol (Fisher Scientific, cat. no. BP2618-500) **! CAUTION** Isopropanol is flammable and irritant. Keep it away from fire. Wear a lab coat and gloves when handling it.
- LB broth (Millipore, cat. no. 71751)
- LB agar (BD, cat. no. 244510)
- Lysozyme from chicken egg white (Sigma-Aldrich, cat. no. L6876)
- Methanol (Fisher Scientific, cat. no. BP1105-4) **! CAUTION** Methanol is toxic and flammable. Keep it away from fire. Wear a lab coat and protective gloves when handling it.
- Milli-Q water (produced by a Milli-Q Synthesis water system with a resistivity of ≥18.2 MΩ·cm)
- *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED; Sigma-Aldrich, cat. no. T7024) **! CAUTION** TEMED causes severe digestive and respiratory tract burns. Use this reagent in a flow hood. Wear a lab coat and protective gloves when handling it.
- NEB 5-α competent *E. coli*, high efficiency (NEB, cat. no. C2987I)
- NEB high efficiency T7 Express $I^q$ competent *E. coli* cells (NEB, cat. no. C3016I)
- NotI (NEB, cat. no. R0189S)
- Nuclease-free water (Life Technologies/Ambion, cat. no. 4387936)
- p19-F primer: 5′-ATGGAACGAGCTATACAAGGA-3′
- pGEX-4T-1-p19-T7 plasmid (Addgene, cat. no. 46306)
- pGEX-3′-Seq primer: 5′-CCGGGAGCTGCATGTGTCAGAGG-3′
- Phusion high-fidelity PCR master mix with HF buffer (NEB, cat. no. M0531S)
- Potassium chloride (KCl; Sigma-Aldrich, cat. no. P5941)
- Pyrogent gel clot LAL single test vials 0.25 EU $ml^{-1}$ sensitivity (Lonza, cat. no. N189-25)
- QIAprep spin miniprep kit (Qiagen, cat. no. 27106)
- QIAquick PCR purification kit (Qiagen, cat. no. 28106)
- SacI (NEB, cat. no. R0156S)
- SalI (NEB, cat. no. R0138S)
- siRNA marker (NEB, cat. no. N2101S)
- Sodium acetate (Sigma-Aldrich, cat. no. S5636)
- Sodium chloride (NaCl; Sigma, cat. no. S5886)
- Sodium hydroxide (NaOH; Sigma-Aldrich, cat. no. S5881) **! CAUTION** Sodium hydroxide is corrosive. Wear a lab coat and protective gloves when handling it.
- Sodium phosphate dibasic heptahydrate ($Na_2HPO_4{\cdot}7H_2O$; Sigma-Aldrich, S9390)
- Sodium phosphate monobasic ($NaH_2PO_4$; Sigma-Aldrich, cat. no. S5011)
- SYBR-Gold (Life Technologies/Invitrogen, cat. no. S-11494)
- T4 DNA ligase (NEB, cat. no. M0202L)
- Triton X-100 (Sigma-Aldrich, cat. no. T8787)
- XhoI (NEB, cat. no. R0146S)

### EQUIPMENT

- Syringe filters, 0.22 µm (Corning, cat. no. 431229)
- Centrifuge tube filters, 0.22 µm (Sigma-Aldrich, cat. no. CLS8160)
- Durapore membranes, 0.45 µm (Millipore, cat. no. HVLP04700)
- Refrigerator, 4 °C (Kenmore, model 253.60722006)
- Freezer, –20 °C (Kenmore, model 253.26722100)
- Freezer, –80 °C (Harris, HLT-25V-85D14)
- Agarose gel electrophoresis system (Thermo Fisher, model EasyCast)
- Anion-exchange column (Agilent Technologies, model Bio WAX NP5)
- Autoclave sterilizer (Getinge Group, model 400/500Ls)
- BLAST web tool (http://blast.ncbi.nlm.nih.gov/)
- Chromas lite (http://technelysium.com.au/?page_id=13)
- Electronic pipette (Integra, model Pipetboy acu)
- FIBERLite rotor F13-14 × 50cy for 50-ml tube (Thermo Scientific, cat. no. 46922)
- Floor centrifuge (Thermo Scientific/DuPont Sorvall, model RC5B)
- Gel imaging system (Carestream, Gel Logic model 212 pro)
- Glass plates for PAGE gels, 1.5 mm spacer (Bio-Rad, cat. no. 165-3312; short plate, cat. no. 165-3308)
- HPLC system (optional and alternative to PAGE; Agilent Technologies, model 1260 Infinity Quaternary LC system; see INTRODUCTION and PROCEDURE)

- Incubation shaker (INFORS HT, model Multitron Standard)
- Incubator (Nuaire, model GP AutoFlow)
- Magnetic stirrer (Corning, model PC-410D)
- Magnetic stirring bar (VWR, cat. no. 74950-290)
- Microcentrifuge tubes, 1.5 ml (Axygen, cat. no. MCT-150-C)
- Microtip 3.2-mm diameter (Misonix, model 419)
- Milli-Q Synthesis water system (Millipore)
- Nalgene polysulfone reusable bottle-top filters (Thermo Scientific, cat. no. DS0320-2545)
- NanoDrop 1000 spectrophotometer (Thermo Scientific)
- NEBcutter (http://tools.neb.com/NEBcutter2/)
- PAGE system (Bio-Rad, Mini-PROTEAN Tetra Cell)
- PCR tubes (Applied Biosystems, cat. no. N8010580)
- pH meter (Beckman, model Φ350)
- Power supply for electrophoresis (Amersham Bioscience, model EPS301)
- Serological pipette (Cellstar, cat. no. 607180)
- Sonicator (Misonix, model S-4000)
- Spectrophotometer (Beckman, model DU530)
- Syringe (BD, 10 ml, cat. no. 309604; 60 ml, cat. no. 309654)
- Tabletop centrifuge (Eppendorf, model 5415D)
- Thermomixer compact (Eppendorf, cat. no. 5350000.013)
- Tube rotator (Thermo Scientific/Labquake, cat. no. 4002110Q)
- Tubes (Corning, 15 ml, cat. no. 430791; 50 ml, cat. no. 430829)

**REAGENT SETUP**

**TBE polyacrylamide gel, 15% (wt/vol)** Use a vertical gel system for casting a polyacrylamide gel (we used the Mini-PROTEAN Tetra Cell system from Bio-Rad). Use a 1.5-mm-thick spacer plate and assemble the gel cast. Prepare 12 ml of 15% (wt/vol) acrylamide solution by adding the reagents in the following table in order. Mix well and pour the acrylamide solution into the gel cast; then add a ten-well comb. Wait for 30 min until the gel is formed. Freshly prepare this gel before use.

| Reagent | Volume |
|---|---|
| Nuclease-free water | 6.18 ml |
| TBE, 10× | 1.2 ml |
| Acrylamide/bis solution, 40% (wt/vol), 19:1 | 4.5 ml |
| TEMED | 5 µl |
| Ammonium persulfate, 10% (wt/vol) | 120 µl |

**Ammonium persulfate, 10% (wt/vol)** Dissolve 100 mg of ammonium persulfate in 1 ml of nuclease-free water. Freshly prepare this reagent before use.

**Antibiotic stock solutions** For ampicillin or carbenicillin stock solution (100 mg $ml^{-1}$), dissolve 1 g of ampicillin sodium salt or carbenicillin disodium salt in 10 ml of nuclease-free water. Sterilize the solution by passing it through a 0.22-µm syringe filter with a 10-ml syringe. Divide the solution into aliquots. The antibiotic stock solutions can be stored at −20 °C for 12 months.

**Bacterial growth media** For LB medium, add 10 g of LB broth to 500 ml of $dH_2O$, mix and autoclave. For LB agar plates, add 20 g of LB agar to 500 ml of $dH_2O$, mix and autoclave. Regardless of whether ampicillin or carbenicillin is used, add antibiotic stock solution to LB medium (to a final concentration of 50 µg $ml^{-1}$) immediately before use or to the plates before pouring LB agar (to a final concentration of 50 µg $ml^{-1}$, allowing the medium to cool to ~50 °C before adding antibiotics). Both medium and plates can be stored at 4 °C for 6 months.

**DNA template** Target sequence can be PCR amplified by using either genomic DNA or cDNA obtained from the cells expressing the target gene as template. If the target sequence does not contain an intron, the user can use genomic DNA to amplify the DNA fragments of target sequence. Genomic DNA can be extracted with commercially available kits from tissues or cell lines. Total RNAs can also be extracted with commercially available kits from tissues or cell lines. RNAs can be converted to cDNA using reverse transcriptase with random hexamer or poly(dT) oligonucleotides as primers according to the manufacturer's protocol.

**EDTA (0.5 M (pH 8.0))** Add 29.2 g of EDTA to 160 ml of Milli-Q water. While stirring the mixture with a stir bar, add NaOH pellets until the pH is 8.0, and allow EDTA to dissolve completely at this pH. Bring the volume to 200 ml. Sterilize the EDTA by autoclaving it. EDTA can be stored at room temperature (~21 °C) for up to 6 months.

**Ethanol, 75% (vol/vol)** Add nuclease-free water to 37.5 ml of ethanol and bring the solution to a final volume of 50 ml.

**HPLC buffer A (25 mM Tris-HCl (pH 7.0) and 2 mM EDTA)** Adding 12.5 ml of 1 M Tris-HCl (pH 7.0) and 2 ml of 0.5 M EDTA to 400 ml of HPLC-grade water. Adjust the volume to 500 ml by adding HPLC-grade water. Filter the buffer using a reusable bottle-top filter device with a 0.45-µm Durapore membrane. This buffer can be stored at room temperature for up to 6 months. This item is optional, and it should only be prepared if HPLC purification of pro-siRNAs is planned.

**HPLC buffer B (25 mM Tris-HCl (pH 7.0), 2 mM EDTA and 5 M NaCl)** Follow the same procedure as that for HPLC buffer A, except add 146.1 g of NaCl. This buffer can be stored at room temperature for up to 6 months. Preparation of this item is optional, and it should only be performed if HPLC purification of pro-siRNAs is planned.

**IPTG, 0.5 M** Dissolve 1.19 g of IPTG in 10 ml of nuclease-free water. Sterilize the solution by passing it through a 0.22-µm syringe filter with a 10-ml syringe. Divide the solution into aliquots and store them at −20 °C for up to 12 months.

**Lysozyme, 100 mg $ml^{-1}$ stock** Dissolve 1 g of lysozyme in 10 ml of nuclease-free water. Divide the stock solution into aliquots and store them at −20 °C for up to 6 months.

**Lysis buffer (50 mM phosphate buffer (pH 7.5), 300 mM NaCl, 10 mM imidazole and 1% (vol/vol) Triton X-100)** Dissolve 8.77 g of NaCl and 0.34 g of imidazole in 400 ml of Milli-Q water. Add 5 ml of Triton X-100 and 12.5 ml of 2 M phosphate buffer (pH 7.5), and bring the volume to 500 ml. This buffer can be stored at 4 °C for up to 12 months. Add lysozyme to reach a concentration of 1 mg $ml^{-1}$ (from 100 mg $ml^{-1}$ stock) immediately before use (see PROCEDURE).

**Potassium chloride, 2 M** Dissolve 7.455 g of KCl in 50 ml of Milli-Q water. Sterilize by autoclaving and store it at room temperature for up to 12 months.

**Phosphate buffer stock solution, 2 M, pH 7.5** Dissolve 23.4 g of $NaH_2PO_4$ in 100 ml of Milli-Q water to prepare solution A (2 M $NaH_2PO_4$), and dissolve 53.61 g of $Na_2HPO_4.7H_2O$ in 100 ml of Milli-Q water to prepare solution B (2 M $Na_2HPO_4$). Combine 16 ml of solution A with 84 ml of solution B. Sterilize the stock solution by autoclaving it. This solution can be stored at room temperature for up to 12 months.

**SDS elution buffer (50 mM phosphate buffer (pH 7.5), 100 mM NaCl and 0.5% (wt/vol) SDS)** Add 200 µl of 5 M NaCl, 250 µl of 2 M phosphate buffer (pH 7.5) and 500 µl of 10% (wt/vol) SDS to 9.05 ml of nuclease-free water. This buffer can be stored at room temperature for up to 12 months.

**Sodium acetate, 3 M pH 5.0** Dissolve 12.3 g of sodium acetate in 40 ml of nuclease-free water. Adjust the pH of the solution to 5.0 with glacial acetic acid. Bring the final volume of the solution to 50 ml. Pass the solution through a 0.22-µm syringe filter with a 60-ml syringe to sterilize. This reagent can be stored at −4 °C for up to 6 months.

**Sodium chloride stock solution, 5 M** Dissolve 146.1 g of NaCl in 500 ml of Milli-Q water, and sterilize by autoclaving it. This solution can be stored at room temperature for up to 12 months.

**Sodium chloride, 0.3 M** Use 600 µl of 5 M NaCl and 9.4 ml of nuclease-free water to prepare 10 ml of 0.3 M NaCl. This solution can be stored at room temperature for up to 12 months. Preparation of this item is optional, and it should only be performed if PAGE-based purification of pro-siRNAs is planned.

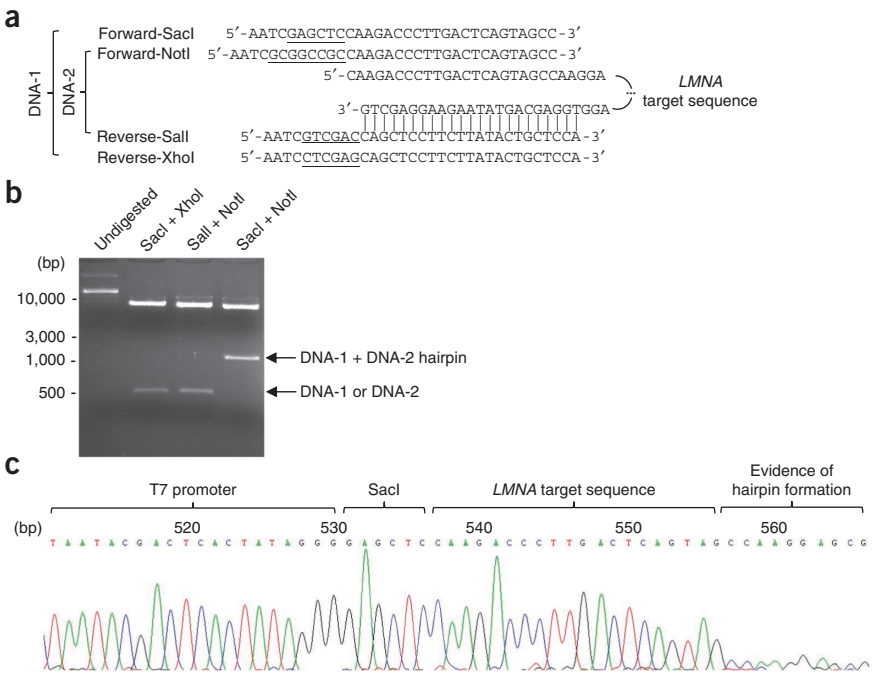


**Figure 2** | Design and analysis of pro-siRNA plasmid. (**a**) Primer design for the *LMNA* pro-siRNA plasmid. Forward primer sequence is complementary to the first 22 nt and the reverse primer sequence is complementary to the last 23 nt of the target sequence. Appropriate restriction site (underlined) and extra 5′-AATC were added to each primer. Brackets indicate the pairs of primers that will amplify DNA-1 fragment and DNA-2 fragment. (**b**) The restriction enzyme digestion test for the *LMNA* pro-siRNA plasmid. Digested plasmids were separated on a 1.2% (wt/vol) agarose gel stained with ethidium bromide. The *LMNA* target sequence is 523 bp. Arrows indicate the digested band and their identities. (**c**) Sequencing traces of *LMNA* pro-siRNA plasmid using p19-F as the sequencing primer (511–565 bp shown). Sequencing traces were visualized using Chromas lite software (http://technelysium.com.au/?page_id=13).

## PROCEDURE

### Engineering the pro-siRNA plasmid

● TIMING 7 d

**1|** Choose a target sequence of more than 200 bp from the target gene of interest, and then design forward and reverse primer sequences (20–25 nt) that will amplify the target DNA segment. To start with, we recommend choosing the sequences immediately after the ATG start site. Use NEBcutter (http://tools.neb.com/NEBcutter2/) to make sure that your target sequence does not contain any of the four restriction sites recognized by SacI, XhoI, SalI or NotI. Use BLAST (http://blast.ncbi.nlm.nih.gov/) to make sure that no genes are present that contain homologous 21-nt sequences, which might, therefore, be targeted inadvertently.

**2|** Synthesize two forward primers, one with a SacI recognition site at its 5′ end and the other with a NotI recognition site at its 5′ end. In addition, synthesize two reverse primers, one with a XhoI recognition site at its 5′ end and the other with a SalI recognition site at its 5′ end. We recommend adding four extra nucleotides (e.g., AATC) at the 5′ end of both forward and reverse primers to facilitate restriction enzyme cutting. An example (lamin A/C (*LMNA*) pro-siRNA plasmid[20]) to illustrate primer design is shown in **Figure 2a**.

**3|** Use a high-fidelity Taq polymerase (we used Phusion high-fidelity PCR master mix) and an appropriate DNA template (DNA or cDNA from cells expressing the target gene, see Reagent Setup) to amplify two DNA fragments: one using the forward primer with a SacI site and the reverse primer with a XhoI site (to obtain the DNA-1 fragment) and the other one using the forward primer with a NotI site and the reverse primer with a SalI site (to obtain the DNA-2 fragment; **Fig. 2a**).

**4|** Recover the two DNA fragments using the QIAquick PCR purification kit. Measure the concentration of DNA using a NanoDrop or UV spectrophotometer.

**5|** Digest 500 ng of pGEX-4T-1-p19-T7 plasmid in a 20-µl reaction volume with 10 units of SacI and XhoI enzymes together at 37 °C for 1–4 h.

**6|** Recover the digested plasmid using the QIAquick PCR purification kit. Measure the concentration of DNA using a NanoDrop or UV spectrophotometer.

**7|** Digest 200 ng of DNA-1 fragment in a 20-µl reaction volume with 10 units of SacI and XhoI enzymes together at 37 °C for 1–4 h.

**8|** Recover the digested DNA-1 with the QIAquick PCR purification kit. Measure the concentration of DNA using a NanoDrop or UV spectrophotometer.

**9|** Ligate 20 ng of digested plasmid with a fivefold molar excess of digested DNA-1 in a 10-µl volume containing 400 units of T4 DNA ligase according to option A or option B. Implementing option B would result in a higher number of successful clones, but it takes longer.

**(A) Conventional ligation**

(i) Incubate the ligation mixture at room temperature for 1–4 h.

**(B) High-efficiency ligation**

(i) Incubate the ligation mixture overnight at 4 °C.

**10|** Use 5 µl of ligation reaction from Step 9 to transform 20 µl of high-efficiency *E. coli* competent cells (we use 5-α competent *E. coli* high efficiency from NEB and transform bacterial cells according to the manufacturer's protocol), and select antibiotic (ampicillin or carbenicillin, see Reagent Setup)-resistant clones on LB agar plates supplemented with 50 µg $ml^{-1}$ antibiotics overnight in a 37 °C incubator.

**11|** Select four antibiotic-resistant *E. coli* colonies, and culture each in 2 ml of LB medium supplemented with 50 µg $ml^{-1}$ antibiotics overnight in a 37 °C shaker at 250 r.p.m. Extract the plasmids using a QIAprep spin miniprep kit. Measure the concentration of DNA using a NanoDrop or UV spectrophotometer.

**12|** To select positive clones, digest 200 ng of each plasmid using SacI and XhoI enzymes and analyze the products by agarose gel electrophoresis (1.2% (wt/vol) in TBE buffer) stained with ethidium bromide. The digested band should be of the same size as the target sequence. If the insert is of the expected size, sequence the plasmid with a pGEX-3′-Seq primer using the standard Sanger sequencing method (we used a commercial sequencing service) to confirm that the inserted sequence is correct.

**13|** Digest 500 ng of pGEX-4T-1-p19-T7 plasmid with the DNA-1 insert with 10 units of SalI and NotI enzymes together for 1–4 h at 37 °C.

**14|** Recover the digested plasmid using a QIAquick PCR purification kit. Measure the concentration of DNA using a NanoDrop or UV spectrophotometer.

**15|** Digest 200 ng of DNA-2 fragment in a 20-µl reaction volume with 10 units of SalI and NotI enzymes together for 1–4 h at 37 °C.

**16|** Recover the digested DNA-2 using a QIAquick PCR purification kit. Measure the concentration of DNA using a NanoDrop or UV spectrophotometer.

**17|** Ligate 20 ng of digested plasmid with a fivefold molar excess of digested DNA-2 in a 10-µl volume containing 400 units of T4 DNA ligase overnight at 4 °C.

▲ **CRITICAL STEP** Overnight ligation is required because the number of positive clones containing the hairpin RNA construct might be low.

**18|** Implement the procedure detailed in Step 10 using 5 µl of the ligation reaction from Step 17.

**19|** Implement the procedure described in Step 11, but culture 12 colonies instead of four.

▲ **CRITICAL STEP** Because long hairpin sequences can be eliminated during plasmid replication, the number of clones containing the full hairpin could be low. It is important to test more clones than would normally be needed for conventional cloning.

**20|** Digest 200 ng of each plasmid with SacI and NotI enzymes, and analyze the digestion by agarose gel electrophoresis (1.2% (wt/vol) in TBE buffer) and ethidium bromide staining to identify positive clones with a full-length hairpin of the expected size. The digested band should be twice the size of the target sequence.

**? TROUBLESHOOTING**

**21|** Perform a quality control test on the pro-siRNA plasmid. Digest three 200-ng aliquots of a plasmid, which migrates with the size expected of the full-length hairpin DNA (from Step 20), separately with three sets of restriction enzymes: set 1, SacI and XhoI; set 2, SalI and NotI; and set 3, SacI and NotI. Analyze the products by agarose gel electrophoresis (1.2% (wt/vol) in TBE buffer) and ethidium bromide staining. The size of the band resulting from the digestion with set 1 and set 2 should be the same as that of the target sequence and that obtained after digestion with set 3 should be about twice that size. As an example, digestion products of the *LMNA* pro-siRNA plasmid are shown in **Figure 2b**.

**22|** Sequence the correct pro-siRNA plasmid (from Step 21) with p19-F primer using the standard Sanger sequencing method (we used a commercial sequencing service). Expect good-quality sequences (with clear and well-separated nucleotide peaks on the sequencing chromatogram) of p19-coding sequences and T7 promoter. Only a few base pairs after the SacI site, the sequencing traces will suddenly drop, suggesting the formation of a hairpin. The good-quality sequences after the SacI site should match the target sequence. Sequencing traces of the *LMNA* pro-siRNA plasmid are shown in **Figure 2c** as an example.

**Transformation of *E. coli* host strain and bacterial culture ● TIMING 1 d**

**23|** Transform the pro-siRNA plasmid into an *E. coli* host strain expressing T7 RNA polymerase. The *E. coli* strain can be any strain that is normally used for the expression of recombinant proteins driven by a T7 promoter; here we use NEB high efficiency T7 Express *Iq* competent *E. coli* cells as a suitable example strain.

**24|** Use a single, freshly transformed *E. coli* colony to inoculate 300 ml of LB medium supplemented with 50 μg $ml^{-1}$ antibiotics in a 1.5-liter flask.

▲ **CRITICAL STEP** Use only freshly transformed *E. coli* colonies. Do not use *E. coli* colonies that have been stored at 4 °C because doing so will reduce the level of pro-siRNA expression.

**25|** Culture the cells by placing the flask in a 37 °C shaker at 200–250 r.p.m. Measure the absorbance at 600 nm every hour until the $OD_{600}$ reaches 0.3–0.6. Thereafter, add 300 μl of 0.5 M IPTG (final concentration 0.5 mM) and continue *E. coli* culture for 1 h.

**26|** Pellet the cells by centrifugation for 10 min at 10,000*g* at 4 °C. Discard the supernatant.

■ **PAUSE POINT** Cell pellet can be stored at –80 °C for 1 month.

**Extraction of pro-siRNAs ● TIMING 1 d**

**27|** Add 10 ml of lysis buffer freshly supplemented with 1 mg $ml^{-1}$ of lysozyme to the *E. coli* pellet. Completely resuspend the pellet by pipetting up and down several times using a 5-ml plastic serological pipette.

**28|** Transfer cell lysate into a 50-ml conical tube, and then rotate the tube in a tube rotator for 30–60 min at 4 °C.

**29|** Sonicate the lysate on ice until the lysate is nonviscous. Settings and processing times vary depending on sonication device used; we use a Misonix S-4000 with a 3.2-mm diameter Microtip and sonicate for 2 min (in 10-s on, 10-s off cycles) at an amplitude of 30.

**30|** Centrifuge the lysate for 30 min at 10,000*g* at 4 °C. Filter the clear lysate through a 0.22-μm syringe filter.

**31|** Incubate the lysate with 1 ml of Ni-NTA resin in a 15-ml conical tube for 4–16 h at 4 °C on a tube rotator.

**32|** Wash the Ni beads (Ni-NTA resin used in Step 31) by centrifuging for 5 min at 500*g* at 4 °C. Discard the supernatant, add 5 ml of lysis buffer and resuspend the beads by inverting the tube several times. Incubate the suspension obtained for 10 min at 4 °C on a tube rotator.

**33|** Repeat Step 32 three more times.

**34|** Transfer the Ni beads into a 1.5-ml microcentrifuge tube and resuspend the beads in 500 μl of SDS elution buffer by inverting the tube several times. Incubate the tube at room temperature for 10 min on an Eppendorf thermomixer at 500 r.p.m.

**35|** Centrifuge the beads at 500*g* for 1 min at room temperature. Transfer the supernatant to a clean microcentrifuge tube.

**36|** Repeat Steps 34 and 35 once more and combine both supernatants.

**37|** Pass the eluate (combined supernatants from Step 36) through a 0.22-μm centrifuge tube filter.

■ **PAUSE POINT** Eluate can be stored at –20 °C for 12 months.

**HPLC purification of pro-siRNAs ● TIMING 3–5 h**

▲ **CRITICAL** The purification procedure that follows can only be implemented if the researchers have access to HPLC equipment. If this is not the case, pro-siRNAs can be purified via the PAGE-based approach described in **Box 1**.

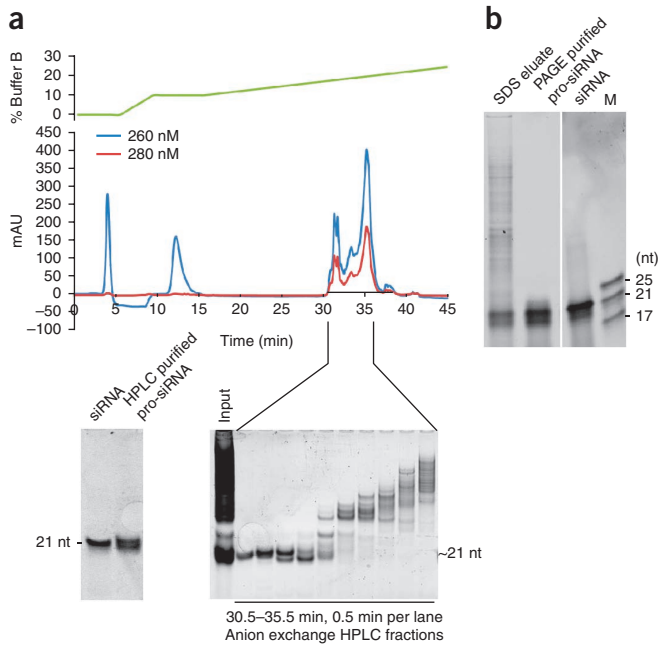


**Figure 3** | Purification of pro-siRNA. (**a**) Anion-exchange HPLC of SDS-eluted *EGFP* pro-siRNAs. Top, the gradient of buffer B (5 M NaCl). Middle, UV 260-nm and 280-nm absorption spectra. Bottom, native PAGE analysis of HPLC fractions (right) stained with SYBR-Gold and pooled fractions (30.5–32.0 min) in final product (left). HPLC fractions were from the indicated period of the HPLC program. (**b**) SDS-eluted RNAs, PAGE-purified pro-siRNA and a synthetic siRNA were separated on a 20% (wt/vol) native polyacrylamide gel stained with SYBR-Gold. M, marker.

**38|** (Optional) If the eluate sample from Step 37 was stored at –20 °C, thaw on ice and centrifuge it for 5 min at 16,000*g* at 4 °C. Please note that a fresh eluate sample from Step 37 can be directly used for HPLC purification.

**39|** Transfer 1 ml of SDS eluate into an HPLC sample tube. Put the sample tube into an autosampler and start the HPLC program (Steps 40–49). Monitor UV absorption at 260 nm and 280 nm. Please note that we used an Agilent 1260 Infinity Quaternary LC system and a Bio WAX NP5 anion-exchange column for this procedure.
▲ **CRITICAL STEP** The HPLC setup and the program detailed in the steps that follow are what we typically use. The conditions provided here could serve as a guideline for other similar HPLC systems and columns, but users should optimize their own HPLC methods when using different systems.

**40|** Set the flow rate to 1 ml $min^{-1}$ and load 1 ml of sample onto the column.

**41|** Wash the column with 100% HPLC buffer A for 6 min.

**42|** Start the first linear gradient of 0–10% buffer B over 4 min.

**43|** Wash the column with 10% buffer B for 6 min.

**44|** Change the flow rate to 0.5 ml $min^{-1}$.

**45|** Start the second linear gradient of 10–25% buffer B over 30 min.
▲ **CRITICAL STEP** pro-siRNAs will be eluted within this gradient after ~14 min.

**46|** Collect samples every 0.5 min per tube beginning at ~14 min (or when RNAs begin to be eluted as indicated by an increase in 260 nM and 280 nM absorption with the 260/280 ratio of ~2).

**47|** Change the flow rate to 1 ml $min^{-1}$.

**48|** Wash the column with 100% buffer B for 5 min.

**49|** Wash the column with 100% buffer A for 8 min.

**50|** Test HPLC fractions: analyze 10 µl of each collected sample in Step 46 on a 15% (wt/vol) TBE polyacrylamide gel (see Reagent Setup) stained with SYBR-Gold (**Fig. 3**). Select and combine the fractions that contain predominantly ~21-nt-long siRNAs.
▲ **CRITICAL STEP** It is crucial to determine the exact retention time when ~21-nt-long pro-siRNAs begin to elute when purifying a pro-siRNA for the first time. In our experience, the retention time and UV absorption patterns of each specific pro-siRNA are highly reproducible. Thus, once elution conditions are determined for a specific pro-siRNA, it may not be necessary to do this test for every HPLC run.
**? TROUBLESHOOTING**

**51|** Precipitate pro-siRNAs: add 500 µl of isopropanol to each 500 µl of combined HPLC fraction in a 1.5-ml microcentrifuge tube, and incubate the mixture at –20 °C for over 1 h. Ten micrograms of glycogen can be added as a carrier for precipitation.

**52|** Centrifuge the samples for 30 min at 16,000*g* at 4 °C. Carefully discard most of the supernatant by aspiration or pipetting.
▲ **CRITICAL STEP** The pellet could be small or invisible, so it is essential to remove the supernatant carefully so that the pellet is not disturbed. Never decant the supernatant. The same precautionary principles should be applied to other RNA isolation steps.

**53|** Add 1 ml of 75% (vol/vol) ethanol and invert the tube several times to wash the pellet. The pellet should be more visible after adding 75% ethanol, and its color should be white.
**? TROUBLESHOOTING**

**54|** Centrifuge the samples for 5 min at 16,000*g* at 4 °C. Carefully discard the supernatant by aspiration or pipetting.

**55|** Centrifuge the samples for another 2 min at 16,000*g* at 4 °C and remove any remaining liquid using a 200-µl pipette tip.

**56|** Open the caps of the tubes to air-dry the pellets for 5 min.

**57|** Dissolve each pro-siRNA pellet in 20 µl of nuclease-free water and combine the resulting solutions.

**58|** Measure the concentration of pro-siRNAs using a NanoDrop or UV spectrophotometer. Test endotoxin contamination using a Limulus amoebocyte lysate (LAL) gel clot assay (Pyrogent gel clot LAL single test vial).
**? TROUBLESHOOTING**

**? TROUBLESHOOTING**
Troubleshooting advice can be found in **Table 1**.

**TABLE 1** | Troubleshooting table.

| Step | Problem | Possible reason | Solution |
|---|---|---|---|
| 20 | No plasmid contains the hairpin RNA construct | Ligation in Step 17 did not work efficiently | Make sure the digestions in Steps 13–16 were sufficiently; purifying the digested plasmid and DNA-2 by agarose gel electrophoresis (1.2% (wt/vol) in TBE buffer) might increase the cloning efficiency |
| | | The hairpin was efficiently eliminated during plasmid replication | We suggest changing or shortening the target sequence |
| 50 | No HPLC fractions contain ~21-nt-long pro-siRNAs | HPLC system and column are different from ours, so the elution conditions of pro-siRNA could be different | Set up different linear gradients and test all elution fractions to establish the correct elution conditions; optimize the conditions based on the performance of your particular HPLC system and column; use chemically synthesized unmodified siRNAs to calibrate system |
| 53, 58 and **Box 1** (steps 4, 18) | No visible RNA pellet; pro-RNA concentration is low | The amount of RNA was too small and the pellet was lost during removal of supernatant | Add glycogen as carrier during the precipitation step; carefully remove the supernatant immediately after each centrifugation |
| **Box 1** (step 13) | Too much RNA staining signal and no distinctive ~21-nt-long pro-siRNA band | Gel was overloaded | Load less RNA in each well |
| 58 and **Box 1** (step 18) | Positive result for endotoxin test or immune stimulation is observed after pro-siRNA transfection | Endotoxin was not efficiently removed during purification | For HPLC purification, make sure that reagents and equipment used are not contaminated with endotoxin; for PAGE purification, a further step to remove endotoxin might be necessary (for example, using Detoxi-Gel endotoxin removing gel); it might be possible to use nontargeting 2′-O-Me RNA oligo (co-transfected with pro-siRNA) to block or reduce immune stimulation[35] |

● TIMING

Steps 1–22, engineering the pro-siRNA plasmid: 7 d
Steps 23–26, transformation of *E. coli* host strain and bacterial culture: 1 d
Steps 27–37, extraction of pro-siRNAs: 1 d
Steps 38–58, HPLC purification of pro-siRNA: 3–5 h
**Box 1**, pro-siRNA purification by native PAGE: 1 d

## ANTICIPATED RESULTS

Expect the yield of purified pro-siRNAs to be in the tens of micrograms per liter of bacterial culture. In our hands, the average yield of several different pro-siRNA constructs and preparations was 42 micrograms per liter and varied between 10 and 80 micrograms per liter[20]. We suspect that tweaking the bacterial culture conditions (for example, using different IPTG concentrations or changing the induction time), as often practiced for optimizing the yield of recombinant proteins, might increase the yield of individual pro-siRNAs.

Purified pro-siRNAs should be mainly 19–22 nt in size, and no obvious sign of contamination with larger or smaller RNA species should be evident. When analyzed by PAGE (**Fig. 3**), pro-siRNAs should migrate close to 21-nt RNA markers under both native and denaturing conditions. Because of their size and sequence heterogeneity, pro-siRNAs may appear as either one slightly smeary band or as multiple bands on polyacrylamide gels.

Purified pro-siRNAs can immediately be used for transfection of human cell lines or other user applications. Because pro-siRNAs are a mixture of heterogeneously sized siRNAs, it is impossible to calculate their exact molecular weight. To calculate molar concentrations, we use an arbitrary value of 12,500 Da as the approximate molecular weight of each pro-siRNA, which is close to the average molecular weight of 21-nt-long synthetic siRNA (~13,300 Da). When pro-siRNAs are efficiently transfected at a concentration of 20 nM, we expect the mRNA level of the target gene, measured by quantitative reverse-transcription PCR (qRT-PCR) and normalized to *GAPDH* expression, to be reduced to at most 10% of the level in negative control siRNA-transfected cells within 24 h. Protein knockdown, typically measured by immunoblotting or flow cytometry, should be evident within 48 h. The stability and/or the half-life of the target protein can influence the level and/or the kinetics of protein knockdown.

Users should explore different concentrations of pro-siRNA in pilot experiments and choose the lowest possible concentration (e.g., 1–5 nM) to minimize the risk of off-target effects, which increase with the concentration of transfected siRNAs[34]. However, for any specific cell line and transfection conditions (choice of transfection method, amount of transfection reagent and cell numbers), transfection may need to be optimized to achieve good knockdown with single-digit nanomolar concentrations of pro-siRNA.



**ACKNOWLEDGMENTS** We thank J. Carrington (Donald Danforth Plant Science Center) for providing the p19 clone. We also thank J. Jin and L. McReynolds from New England Biolabs and P. Deighan and E. Kiner from Harvard Medical School for assistance and advice in establishing this method. We also thank Lieberman Laboratory members for technical assistance. This work was supported by a US National Institutes of Health grant (no. AI087431; J.L.) and a GlaxoSmithKline(GSK)-Immune Disease Institute(IDI) Alliance fellowship (L.H.).

**AUTHOR CONTRIBUTIONS** L.H. designed the protocol with suggestions from J.L. and L.H., and J.L. wrote the paper.

**COMPETING FINANCIAL INTERESTS** The authors declare no competing financial interests.



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