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Production of endoribonuclease-prepared short interfering RNAs for gene silencing in mammalian cells

Ralf Kittler[1], Anne-Kristin Heninger[1], Kristin Franke[1], Bianca Habermann[2] & Frank Buchholz[1]

[1]Max-Planck-Institute for Molecular Cell Biology and Genetics, Pfotenhauerstrasse 108, D-01307 Dresden, Germany. [2]Scionics Computer Innovation, GmbH, Pfotenhauerstrasse 110, D-01307 Dresden, Germany. Correspondence should be addressed to F.B. (buchholz@mpi-cbg.de).

RNA interference (RNAi) has revolutionized functional genomic studies in many model organisms. In particular, the introduction of short double-stranded RNAs (dsRNAs) as mediators of RNAi in mammalian cells has moved loss-of-function studies in these experimental systems to a new plane[1]. Now the two most commonly used RNAi triggers in mammalian cells are chemically or *in vitro* synthesized short interfering RNAs (siRNAs)[2] and *in vivo* expressed short hairpin RNAs (shRNAs)[3]. We, and others, have pioneered an alternative technology, which is considerably more cost-efficient and less laborious than siRNA and shRNA methodologies. Our method is based on the generation of siRNAs by digestion of long dsRNAs with recombinant *Escherichia coli* RNase III or Dicer[4–8]. The resulting endoribonuclease-prepared siRNAs (esiRNAs) have proven to be efficient and specific mediators of RNAi in mammalian cells[9–11]. Here we describe a robust and simple protocol for the production of esiRNA (Fig. 1): a cDNA fragment tagged with T7 promoter sequence by PCR is transcribed *in vitro* to produce dsRNA. By limited digestion, the long dsRNA is then converted to siRNAs of <30 base pairs (bp) and spin-purified in a single column. Because of its ease, speed and cost-efficiency, this protocol can be easily scaled for the generation of libraries for (sub)genomic screens.

MATERIALS

REAGENTS

Agarose, electrophoresis grade (Invitrogen)
BIOTAQ Red DNA polymerase (Bioline)
Complementary DNA (cDNA) clones or cDNA preparations
Deoxynucleoside triphosphate (dNTP) mix (10 mM; Bioline)
dsRNA digestion buffer (20 mM Tris-HCl, 0.5 mM EDTA, 5 mM $MgCl_2$, 1 mM Diothiothreitol (DTT), 140 mM NaCl, 2.7 mM KCl, 5% (vol/vol) glycerol (pH 7.9))
Elution buffer (20 mM Tris-HCl, 1 mM EDTA, 520 mM NaCl, (pH 8.0))
70% (vol/vol) ethanol, cold
Equilibration buffer (20 mM Tris-HCl, 1 mM EDTA, 300 mM NaCl (pH 8.0))
esiRNA loading dye (3 mg/ml Orange G, 5% (wt/vol) Ficoll; both from Sigma-Aldrich)
GST-RNase III (prepared as previously described[4])
Isopropanol
Marker DNA (for example, 25-bp DNA ladder, HyperLadder V; Bioline)
MEGAscript *in vitro* transcription kit (Ambion)
$MgCl_2$ (50 mM; Bioline)
10× ammonium reaction buffer (160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl (pH 8.8), 0.1% (vol/vol) Tween 20; Bioline)
Primer oligonucleotides containing the T7 promoter sequence, as described in Step 2
Q Sepharose FastFlow (Amersham Biosciences)
Wash buffer, (20 mM Tris-HCl, 1 mM EDTA, 400 mM NaCl (pH 8.0))

EQUIPMENT

96-well filter plates UNIFIL 98-800U for large-scale synthesis (Whatman)
96-well PCR plates (Nerbe)
Empty spin columns (for example, Micro Bio-Spin Chromatography Columns; BioRad Laboratories)
Deep-well plates (1.1 ml) for large-scale synthesis (Nerbe)
Multifuge 4KR, for large-scale synthesis (Kendro)
Silicon seals for deep-well plates (Nerbe)
Thermal cycler programmed with the desired amplification protocols (MJ Research)

Generation of a template for *in vitro* transcription by PCR

PROCEDURE

1| The template for *in vitro* transcription can be generated from a cDNA clone or directly from cDNA using vector- or target-specific primers appended with T7 promoter sequences. *cDNA clones have the advantage that universal vector-specific primers can be used to amplify the insert. Conversely, cDNA sequence-specific primers allow a high degree of flexibility to choose the target region for esiRNA production. For the selection of the esiRNA target region, we recommend using the web server DEQOR[12], which allows the identification of cDNA regions containing a high percentage of efficient silencers and a low number of cross-silencers (**Fig. 2**). The link to the program is available online (http://deqor.mpi-cbg.de). This web server uses accession numbers from GenBank or raw cDNA sequences.*

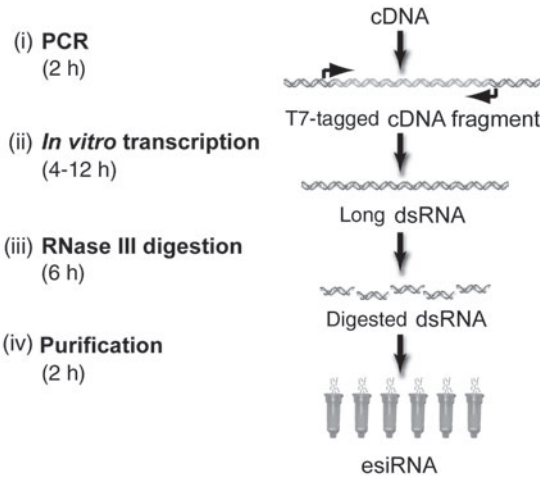

Figure 1 | Flowchart of esiRNA production.

2| After selecting a 400–600-bp esiRNA target region, design primers appended 5′ with the T7 promoter sequence 5′-CGTAATACGACTCACTATAGGGAGAG-3′.
When using cDNA clones with the same vector backbone, a universal primer pair can be used. For primer design we routinely use the Primer3 program with the provided default parameters[13]. The web server for Primer3 is available online (http://frodo.wi.mit.edu).
▲CRITICAL STEP

3| Set up the amplification reaction.

10× NH_4 reaction buffer	5 µl
$MgCl_2$ (50 mM)	2 µl
dNTP mix (10 mM)	4 µl
T7 forward primer (10 µM)	1 µl
T7 reverse primer (10 µM)	1 µl
Template DNA (10 ng/µl plasmid DNA or 100 ng/µl cDNA)	1 µl
BIOTAQ Red polymerase (1 u/µl)	2 µl
Water	34 µl

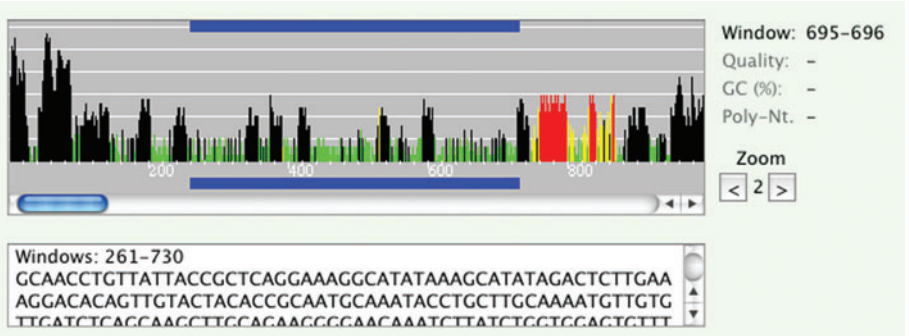

Figure 2 | Selection of an optimal esiRNA target region with DEQOR. The web server DEQOR allows the identification of efficient silencers and cross-silencers using state-of the-art siRNA design criteria. The *in silico* analysis of a given mRNA sequence is graphically shown: efficient silencer windows (green), inefficient silencer windows (black), perfect cross-silencer windows (red) and imperfect cross-silencer windows (yellow). Suitable sequence regions can be dragged (depicted by the blue bar) and copied from a field below containing the marked sequence for subsequent primer design.

4| Amplify the template as follows:

Cycle number	Denaturation	Annealing	Polymerization
1	2 min at 94 °C	30 s at 60 °C	30 s at 72 °C
2–39	30 s at 94 °C	30 s at 60 °C	30 s at 72 °C
40	30 s at 94 °C	30 s at 60 °C	5 min at 72 °C

Annealing and extension times may need to be adapted for individual amplicons.
■ **PAUSE POINT** PCR products can be stored at –20 °C for at least one year.

5| Analyze 3 µl of PCR product on a 1.5% (wt/vol) agarose gel with an appropriate marker.
An intense band should be observed indicating high yield of amplification (typically >100 ng/µl).
▲**CRITICAL STEP**

Generation of long dsRNA by *in vitro* transcription

6| Using components from the MEGAscript kit and the PCR product obtained in Step 5, set up an *in vitro* transcription reaction as follows:

10× T7 reaction buffer	1 µl
Uridine triphosphate (UTP; 75 mM)	1 µl
Adenosine triphosphate (ATP; 75 mM)	1 µl
Guanine triphosphate (GTP; 75 mM)	1 µl
Cytosine triphosphate (CTP; 75 mM)	1 µl
PCR product	4 µl
T7 enzyme mix	1 µl

7| Perform the *in vitro* transcription and annealing reaction in a thermal cycler as follows:

In vitro transcription	4–12 h at 37 °C
Denaturation	3 min at 90 °C Ramp to 70 °C with 0.1 °C/s 3 min at 70 °C Ramp to 50 °C with 0.1 °C/s 3 min at 50 °C
Annealing	Ramp to 25 °C with 0.1 °C/s

Do not freeze the long dsRNA as this will promote the formation of aggregates, which hamper subsequent digestion. This is especially true for dsRNAs of >800 bp.

8| Check 0.5 µl of the product on a 1.5% (wt/vol) agarose gel against an appropriate marker.
A very intense band should be observed indicating high yield of in vitro transcription (4–10 µg/µl). This gel check can be omitted when using the BIOTAQ Red polymerase from Bioline, especially for the production of esiRNAs in 96-well formats. A marker dye included in the polymerase indicates an efficient in vitro transcription reaction in a color shift from red to yellow. This presumably occurs as a result of a change in the pH of the reaction as the NTPs are consumed.
➨ **TROUBLESHOOTING**

Digestion of long dsRNA

9| Set up the digestion reaction as follows: mix 10 µl of *in vitro* transcription product from Step 7 with 90 µl of dsRNA digestion buffer, containing 5 µg GST-RNase III, and mix vigorously.
▲**CRITICAL STEP**

10| Perform the digestion reaction as follows: incubate with agitation for 4 h at 20–25 °C, then shift the temperature to 37 °C for 2 h more.
■ **PAUSE POINT** Digestion products can be stored at –20 °C for at least 1 month.

11| Mix 3 µl of digestion product with 3 µl of esiRNA loading dye and analyze the sample on a 4% (wt/vol) agarose gel against an appropriate marker.
*The 4% agarose gels are somewhat difficult to prepare with a microwave. A good solution is to dissolve the agarose in a water bath set to 95 °C, which takes about 90 min. The digestion product smear should be shorter than 30 bp, whereas most of the smear should range between 18 and 25 bp (**Fig. 3**, lane 2).*
➨ **TROUBLESHOOTING**

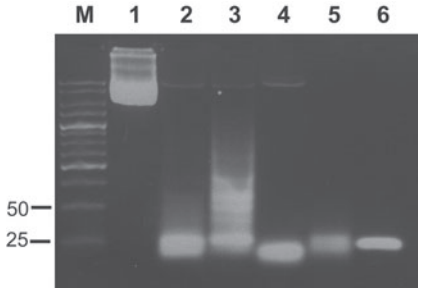

Figure 3 | Digestion of long dsRNA. A cDNA fragment (452 bp) tagged with T7 promoter sequence was *in vitro* transcribed to produce a long dsRNA, which was digested with different amounts of GST–RNase III. The long dsRNA, digestion products, purified esiRNA and a chemically synthesized siRNA (21mer) were separated on a 4% agarose gel. M, 25-bp ladder, the 25- and 50-bp bands are indicated; lane 1, 0.5-µl long dsRNA; lanes 2 and 3, 3 µl of digestion product generated with the 5 µg (recommended) and 2 µg (too little) GST–RNase III, respectively; lane 4, 15 µg (too much) GST–RNase III; lane 5, 500 ng esiRNA purified from the digestion product shown in lane 2; and lane 6, 500 ng of a 21-nt chemically synthesized siRNA.

EsiRNA purification

12| Prepare spin columns for purification as follows: add 200 µl of Q-Sepharose (slurry) to an empty spin column or to a Whatman 96-well filter plate (for large-scale synthesis). Put the column/filter plate in/on an empty 2-ml microcentrifuge tube or deep-well plate. Add 500 µl of equilibration buffer to the column. Centrifuge at 1,000*g* for 1 min and discard the flowthrough. Add another 500 µl of equilibration buffer, centrifuge at 1,000*g* for 1 min and discard the flowthrough.

13| Load all of the digested dsRNA onto the column and incubate for 5 min at 15–25 °C.

14| Centrifuge at 1,000*g* for 1 min and discard the flowthrough.

15| Add 500 µl of wash buffer, centrifuge at 1,000*g* for 1 min and discard the flowthrough.

16| Add 300 µl of elution buffer (270 µl for deep-well plates), centrifuge at 1,000*g* for 1 min and collect the flowthrough in a new microcentrifuge tube or deep-well plate.

17| Repeat Step 16.

18| Add 500 µl of isopropanol (400 µl for deep-well plates), seal and vortex. Store at –20 °C for at least 1 h.
■ **PAUSE POINT** The isopropanol-esiRNA mixture can be stored at –20 °C for at least 3 d.

19| Centrifuge at 16,000*g* for 15 min at 4 °C (or at 4,400*g* for 60 min at 4 °C for deep-well plates). Gently discard the supernatant and wash the pellet with 500 µl of cold 70% (vol/vol) ethanol.
▲**CRITICAL STEP**

20| Centrifuge at 16,000*g* for 5 min at 4 °C (or at 4,400*g* for 5 min at 4 °C for deep-well plates). Gently discard the supernatant and wash the pellet with 500 µl of cold 70% (vol/vol) ethanol.

21| Centrifuge at 16,000*g* for 5 min at 4 °C (or at 4,400*g* for 5 min at 4 °C for deep-well plates). Gently discard the supernatant and dry the esiRNA pellet in a Speed Vac.

22| Dissolve the pellet in 100 µl of water.

23| Mix 3 µl of purified esiRNA with 3 µl esiRNA loading dye and check on a 4% (wt/vol) agarose gel with an appropriate marker.

24| Measure OD_{260} to quantify the esiRNA concentration.
For quantification of esiRNA, we routinely use the extinction factor of dsDNA. The average esiRNA yield should be ~40 µg.

25| Store the esiRNA at –20 °C
Under these conditions esiRNA can be stored for more than 6 months with no detectable reduction in silencing efficiency.

26| The esiRNA can be used directly for the transfection of mammalian cells using appropriate transfection methods, such as lipofection and electroporation.
The amount of esiRNA for each transfection depends on the cell type and the transfection method. For HeLa cells we routinely transfect with Oligofectamine (Invitrogen) 200–400 ng esiRNA/ml medium, that is, 20–40 ng for a single transfection in 96-well plates.

TROUBLESHOOTING TABLE

PROBLEM	SOLUTION
Step 8 There is a low yield of dsRNA (**Fig. 3**, lane 3).	The amount of DNA template was too low. Use at least 250 ng DNA for *in vitro* transcription.
Step 11 The digestion products are too long.	The amount of GST–RNase III was too low or the amount of dsRNA was too high. If the digestion product smear is <50 bp, add 2 μg of GST-RNase III and incubate for another hour at 37 °C. If the digestion product smear is >50 bp, add 4 μg of GST–RNase III and incubate for another hour at 37 °C.
Step 11 The digestion products are too short (**Fig. 3**, lane 4).	The amount of dsRNA was too low (see Troubleshooting for Step 8), or the amount of GST–RNase III was too high. Repeat the digestion with more dsRNA or decrease the amount of GST–RNase III.

CRITICAL STEPS

Step 2 When choosing cDNA clones or designing cDNA-specific primers bear in mind that the amplicon length is critical for the downstream digestion of the dsRNA. We obtained best results with amplicon lengths between 400 and 600 bp. For shorter templates, the streamlining of RNase III digestion conditions seems to be more difficult, and for templates longer than 800 bp, we occasionally observe problematic annealing between the two single-stranded RNAs.

Step 5 The amount of PCR product is limiting for the yield of *in vitro* transcription. Therefore at least 250 ng of DNA should be used as template for a 10-μl *in vitro* transcription reaction. We obtain our highest yields using >400 ng DNA.

Step 9 The amount of GST–RNAse III is critical for optimal digestion. The recommended amount of 5 μg GST–RNase III is optimal for the digestion of 30–100 μg dsRNA. Under the digestion conditions described in Step 10, 90% of all samples should have digestion product smears of <30 bp. Because the specific activity of GST–RNase III may differ between preparations, we recommend performing an initial titration of each enzyme stock to ensure optimal digestion results.

Step 19 Care should be taken not to lose the pellet when decanting isopropanol after centrifugation. The same applies to Steps 20 and 21.

COMMENTS

RNAi-based gene function analyses in mammalian cells require transient or stable knockdowns depending on protein half-life or the onset of downstream effects. shRNA expression vectors are the method of choice for persistent knockdown studies and positive selection screens[3]. For many experiments, however, transient gene silencing is desirable. The most commonly used mediator for transient RNAi is chemically synthesized siRNAs. The advantages of siRNAs over shRNA expression vectors are that they are more efficiently introduced into cells, are less toxic and offer far greater control over interfering dsRNAs at the cellular level. One major drawback of chemically produced siRNAs, however, is their high cost. The protocol described here for the production of esiRNAs offers a very fast and cost-efficient alternative. Initial comparative studies between siRNA and esiRNAs showed similar silencing efficiencies, with a knockdown efficiency of >70% at the mRNA level for >90% of all tested esiRNAs (R.K and F.B., unpublished data). EsiRNA technology is

especially useful for the rapid generation of large libraries. Using the described protocol we have previously generated a library of ~15,000 human esiRNAs[10] and another library of ~25,000 mouse esiRNAs (R.K and F.B., unpublished data).

SOURCE

This protocol is based on new developments to Buchholz, F., Drechsel, D., Ruer, M. & Kittler, R. Production of siRNA *in vitro* by enzymatic digestion of double-stranded RNA in *Gene Silencing by RNA: Technology and Application* (ed. Sohail, M.) 87–99 (CRC Press, Boca Raton, Florida, USA, 2004). For further discussion on the delivery of siRNA into cells and its subsequent effects, see Hannon, G.J. *RNAi: A Guide to Gene Silencing*. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA, 2003; http://www.cshlpress.com/link/rnaip.htm).

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