Gene Silencing by RNA Interference

Technology and Application

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6 Production of siRNA *In Vitro* by Enzymatic Digestion of Double-Stranded RNA

Frank Buchholz, David Drechsel, Martine Ruer, and Ralf Kittler

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6.1 INTRODUCTION

RNAi is an ancient defense and regulatory mechanism to silence genes in a variety of organisms, including plants, insects and vertebrates (reviewed in references[1,2]). The discovery of this mechanism has enabled researchers to utilize it as a tool for gene function analyses in model organisms. Its relative ease has generated a wealth of new information, and for some organisms whole genome RNAi analyses have been accomplished.[3–5] In many species, long dsRNA can be used to trigger RNAi when introduced into the cytoplasm of a cell. However, long dsRNAs have a nonspecific negative effect on cell proliferation in many vertebrate cells because they trigger an interferon response (reviewed inreference [6]). Interferon production activates two pathways in the cell that negatively affect cell proliferation. First, interferons cause the activation of dsRNA-dependent protein kinase (PKR), leading to the general inhibition of protein production by phosphorylation of the translation factor eIF-2α. Second, 2'-5' oligoadenylate synthetase (2'-5'-OAS) is turned on, leading to mRNA degradation via activation of RNase L. As a consequence, long dsRNA is not useful for specific gene silencing in most mammalian cells.

Detailed analyses of the RNAi pathway revealed that long dsRNA is processed into ca. 20 to 25 bp fragments within cells by an RNase III-like endoribonuclease called Dicer.[7–10] These short dsRNA fragments are termed short interfering RNA (siRNA) and contain 2-nt 3'-end overhangs, the signature of all RNase III enzymes.[11] Interestingly, siRNA does not trigger a strong interferon response in mammalian cells

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while efficiently and specifically acting as a silencing trigger for a corresponding gene.[12]

Shortly after the discovery of siRNAs as the effective molecules in RNAi, several groups demonstrated that either chemically synthesized, *in vitro* transcribed siRNAs or short hairpin RNAs (shRNAs) can be used for gene knockdown studies in mammalian cells (reviewed in reference [13]). While these methods for gene silencing are very useful, they all have major limitations, especially for functional genomic studies. First, these methods of generating siRNA reagents are expensive and labor intensive. Second, each siRNA or shRNA has a different potential to silence the mRNA of a corresponding gene. Some rules have been established that help to design siRNA or shRNA molecules.[14] However, we still do not understand enough about the silencing mechanism to predict effective silencing molecules at a very high rate. Therefore, each siRNA or shRNA has to be tested for its effectiveness in knocking down the gene of interest, and usually more than one siRNA or shRNA has to be produced and compared.

These drawbacks can be circumvented by using siRNA hydrolyzed *in vitro* from a long dsRNA with purified Dicer[15,16] or other dsRNA-specific endoribonucleases, including RNase III from *E. coli*.[17,18] Both methods are cheap, easy, fast to perform, and can be carried out in any standard molecular biology laboratory. Using this strategy, a pool of different siRNAs are generated that all have the potential to silence the gene of interest. The complex mixture of different siRNAs ensures that at least some effective molecules will be present, thus eliminating the requirement to screen for an active siRNA for each individual gene. We refer to these siRNAs as endoribonuclease prepared siRNAs or, in short, esiRNAs.

While both Dicer and RNase III can be used to generate esiRNAs, both enzymes produce different mixtures of siRNA molecules. Digestion with Dicer starts at the ends of the dsRNA and proceeds in a sequential manner.[12,19,20] As a consequence, this approach produces a set of nonoverlapping siRNAs. In contrast, RNase III from *E. coli* cleaves long dsRNA in a more or less random fashion,[17,21] leading to a more complex mixture of siRNAs. A larger number of different siRNAs may increase efficiency and specificity. A further disadvantage of the Dicer protocol is that the purification of the enzyme is considerably more difficult, and the activity of the enzyme is much lower than the activity of purified RNase III. Due to the high activity and processivity of *E. coli* RNase III, large quantities of esiRNA can be produced with very little enzyme. Much more of the purified Dicer has to be added to achieve the same yield of esiRNA. For both approaches, the digested dsRNAs of the correct size have to be purified away from residual longer dsRNA fragments that would otherwise trigger the interferon response in transfected mammalian cells.

A potential drawback of esiRNA may be the cross-silencing of highly homologous genes. However, previously published data[9] and unpublished data from our own laboratory argue that cross silencing of homologous genes is typically not observed.

A disadvantage of using *E. coli* RNase III for the production of esiRNA is that complete digestion produces dsRNA fragments of 12 to 15 bp, and these fragment sizes are too short to trigger efficient RNAi in mammalian cells. However, conditions can be developed in which the vast majority of the fragments are of a size range that allows efficient gene knockdown in mammalian cells (see also Figure 6.3).

In this chapter we present a protocol for esiRNA preparation from long dsRNA using an *E. coli* GST-RNase III fusion protein. The protocol can be divided into three basic steps that are summarized in Figure 6.1. Some of the materials and reagents are toxic and should be handled with care, taking the appropriate safety measures. Essentially, the three synthesis steps can be conveniently performed within two days. Day one involves preparation of *in vitro* transcription templates by PCR and large-scale synthesis of dsRNA. Limited digestion of dsRNA with GST-RNase III and purification of digestion products can be performed on day two.

6.2 CLONING, EXPRESSION, AND PURIFICATION OF *E. COLI* RNASE III

The coding sequence of *E. coli* RNase III can be amplified from bacterial genomic DNA and cloned into expression vectors. Because it is an endogenous *E. coli* gene, it expresses well and over-expression in its natural host does not produce any obvious

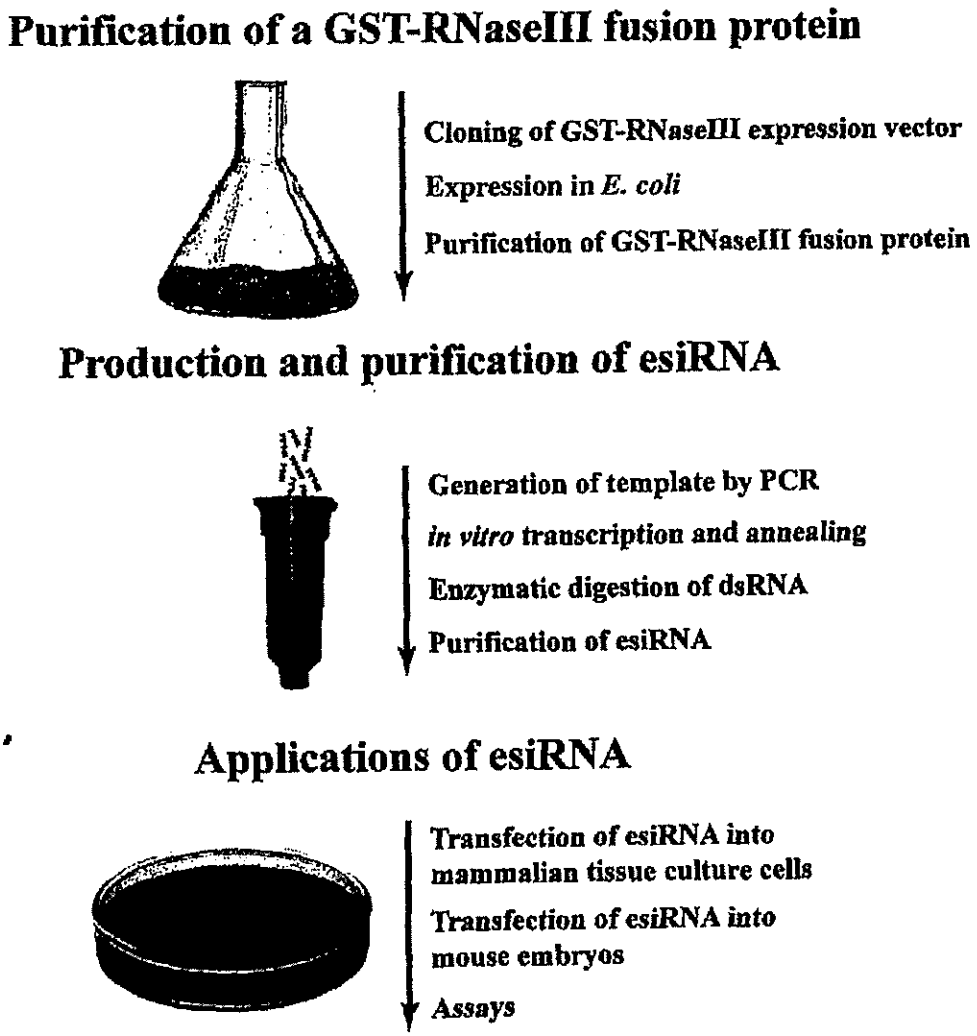

FIGURE 6.1 Overview of RNAi experiments utilizing esiRNAs. Distinct steps in the process are outlined and each step is subdivided into experimental procedures.

In this chapter we present a protocol for esiRNA preparation from long dsRNA using an *E. coli* GST-RNase III fusion protein. The protocol can be divided into three basic steps that are summarized in Figure 6.1. Some of the materials and reagents are toxic and should be handled with care, taking the appropriate safety measures. Essentially, the three synthesis steps can be conveniently performed within two days. Day one involves preparation of *in vitro* transcription templates by PCR and large-scale synthesis of dsRNA. Limited digestion of dsRNA with GST-RNase III and purification of digestion products can be performed on day two.

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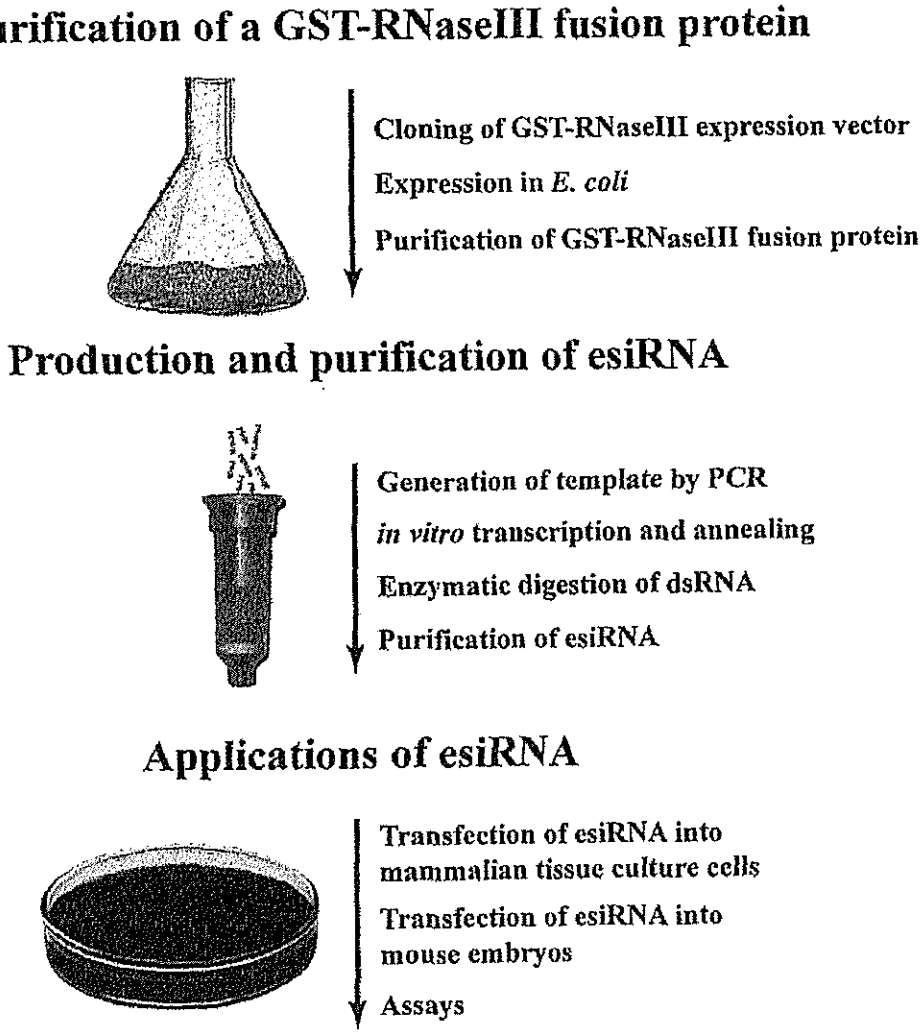

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phenotype in the bacteria. We express *E. coli* RNase III as a GST-fusion protein to allow simple purification of the product. The GST-fusion protein is active without removal of the GST-moiety. In fact, the GST-RNase III fusion protein may alter the cleavage properties toward the generation of longer fragments. This property may serve the efficient production of esiRNAs for use in gene silencing in mammalian cells. Several companies sell purified *E. coli* RNase III. To our knowledge these proteins do not carry a GST-tag. We do not know whether the reaction conditions have to be altered using these proteins for esiRNA production.

PROTOCOLS

Protocol 6.1 Cloning of *E. coli* RNase III

Materials

- *E. coli* genomic DNA (QIAamp DNA Kit, Qiagen)
- Primer RNase III-Bam:
  5'-CGCGGATCCAACCCCATCGTAATTAATCGGCTTCA
- Primer RNase III-SMA: 5'-GACGTCCGACGATGGCAAT
- Taq DNA polymerase and buffers (Bioline)
- Thermal cycler (MJ Research)
- *Bam* HI and *Sma* I (NEB)
- Plasmid pGEX-2T (Amersham Biosciences)
- PCR Purification Kit (Qiagen)

Experimental Procedures

1. Amplify the coding sequence of *E. coli* RNaseIII from bacterial genomic DNA using the primers RNase III-Bam and RNase III-SMA (94°C/3 min; 30 cycles of 94°C/30 sec, 55°C/30 sec, and 72°C/60 sec; final elongation step, 72°C/10 min).
2. Purify the amplified fragment using the PCR purification kit.
3. Digest with *Bam* HI and *Sma* I and clone the fragment into the vector pGEX-2T digested with the same enzymes to produce pGEX-2T-RNase III.
4. Transform into a standard laboratory strain such as DH5α)

Protocol 6.2 Expression and Purification of GST-RNase III

RNase III is naturally present in *E. coli*, and its over-expression does not cause an obvious phenotype. Therefore, large amounts of highly pure enzyme can be expressed and purified in one simple purification step (Figure 6.2).

Materials

- Emulsiflex homogenizer (Avestin)
- Competent *E. coli* BL21 (Novagen)
- 5 × Bradford protein assay reagent (100 mg Coomassie Brilliant Blue G (Merck) 1.02082.0005, dissolve in 50 ml of 95% ethanol; add 100 ml 85.7% (v/v) phos-

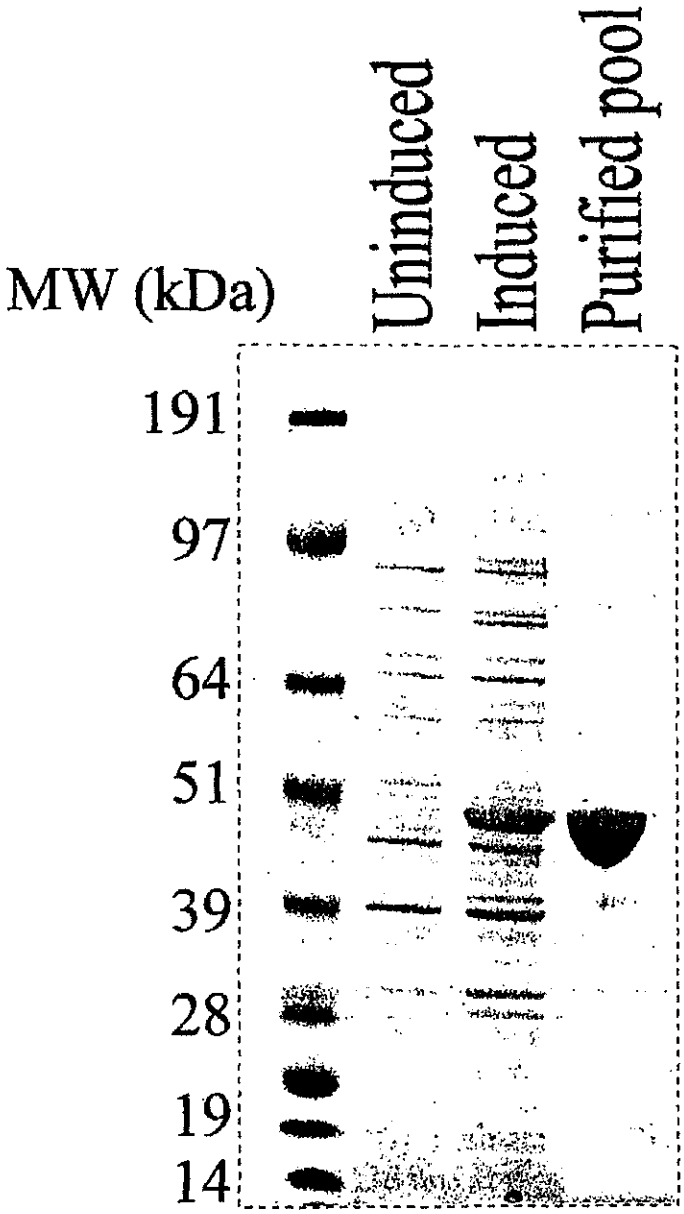
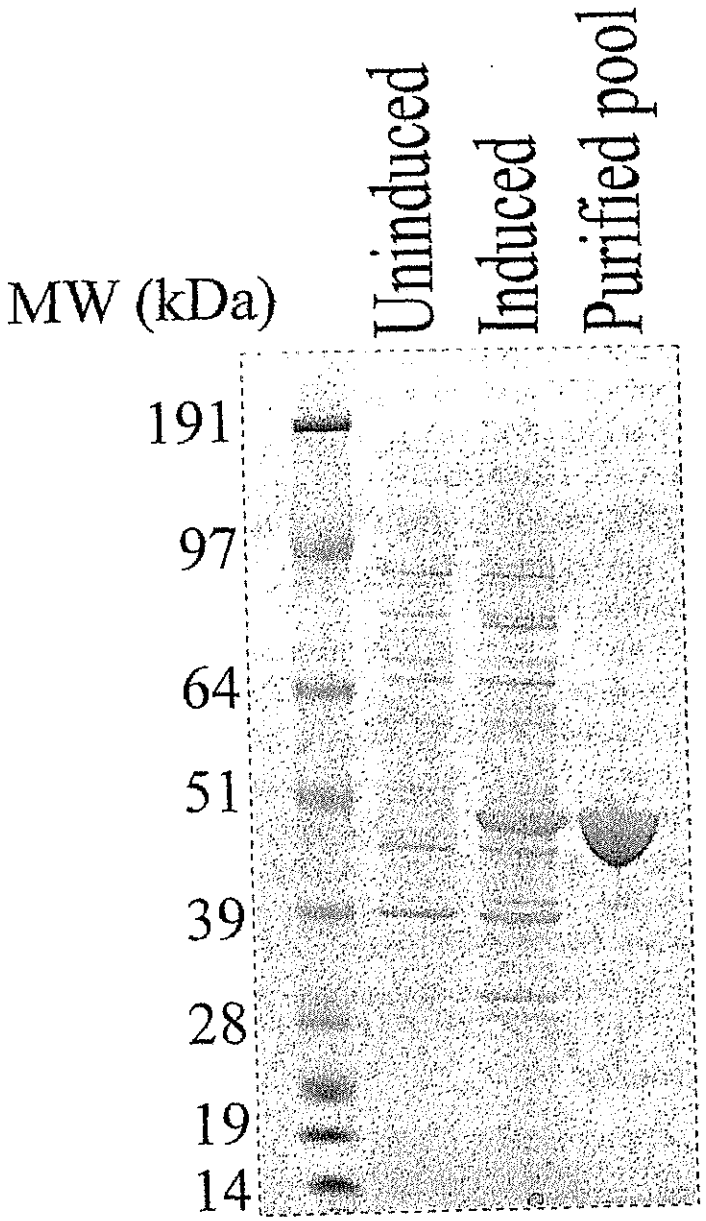


FIGURE 6.2 Purification of the GST-RNase III fusion protein. Samples taken at indicated steps during expression and purification are analyzed by SDS-PAGE stained with Coomasie blue. Uninduced, total cell extract prior to IPTG induction; induced, total cell extract at the end of induction period; purified pool, the final preparation.

phoric acid; mix thoroughly; add water to 200 ml for a 5 × stock; filter through Whatman #1 paper; dilute with H_2O to 1 × solution, which can be stored at 4°C for at least 2 weeks)

- Terrific Broth (TB) (Dissolve in 900 ml deionized H_2O, 12 g tryptone, 24 g yeast extract, and 4 ml glycerol; autoclave and cool to <60°C; add100 ml of 0.17 M KH_2PO_4/0.72 M K_2HPO_4 buffer)
- Luria-Bertani medium (LB) (Dissolve in 900 ml deionized H_2O, 10 g tryptone, 5 g yeast extract, and 10 g NaCl; adjust pH to 7.0 and volume to 1 l with H_2O; autoclave
- 20% (w/v) glucose (20 × stock)



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- 20% (w/v) glucose (20 × stock)

- 100 mg/ml ampicillin (1000 × stock)
- IPTG (Diagnostic Chemicals)
- Lysis buffer (1 × PBS, 0.5% (v/v) Tween-20, 1 mM EDTA, 1 × Protease inhibitors to be added before lysing the cells)
- Column buffer with Tween-20 (1 × PBS, 0.25 M M NaCl, 0.05% (v/v) Tween-20, 0.5 mM DTT)
- Column buffer without Tween-20 (1 × PBS, 0.25 M M NaCl, 0.5 mM DTT)
- Elution buffer (50 mM HEPES, pH 8.0, 300 mM NaCl, 0.5 mM DTT, 10 mM reduced Glutathione; check pH and adjust to 8.0 with NaOH after adding reduced Glutathione from a 250 mM stock in H_2O)
- 2 × Storage buffer (40 mM Tris-Cl, pH 7.9, 1 mM EDTA, 10 mM $MgCl_2$, 2 mM DTT, 280 mM NaCl, 14 mM KCl)
- Glutathione-Sepharose 4 Fast Flow (Amersham, #17-5132-01)

1000 × Protease Inhibitors

Protease inhibitor	Supplier	Specificity	[Final](mg/ml)	Mode of action
Chymostatin	Calbiochem	Cysteine and serine	6	Reversible
Leupeptin	BioMol	Serine and cysteine	0.5	Reversible
antipain-HCl	Sigma-Aldrich	Serine and cysteine	10	Reversible
Aprotinin	Sigma-Aldrich	Serine	2	Reversible
Pepstatin	BioMol	Aspartic	0.7	Reversible
APMSF	Sigma-Aldrich	Serine	10	Irreversible

Prepare a 1000 × stock in dry DMSO (Sigma-Aldrich). Store 200-µl aliquots at –20°C.

Experimental Procedures

Induction of protein overexpression

1. Inoculate a single colony of *E. coli* BL21, freshly transformed with the pGEX-2T RNase III expression plasmid, in 50 ml of LB broth containing 100 µg/ml ampicillin and 1% (v/v) glucose in a 250-ml flask to prepare a preculture.
2. Incubate overnight (12 to 16 hr) at 37°C, with shaking at 180 rpm.
3. Transfer the overnight culture to a 50-ml conical tube, pellet (4000 rpm, 5 min at RT, Heraeus Multifuge 3SR), and resuspend in 50 ml fresh media.
4. Inoculate 20 ml of this washed culture in 1000 ml TB containing 100 µg/ml ampicillin.
5. Incubate at 37°C for 2.5 h and then induce protein expression by adding 0.2 mM IPTG.
6. Incubate with shaking at 37°C for 3 h.

Extract preparation

7. Harvest cells (5000 rpm, 10 min, 4°C, Beckman JLA 8.1000) and resuspend in ice-cold lysis buffer (5 ml lysis buffer/g bacterial pellet) containing 1 × protease inhibitors.
8. Prepare extracts immediately by passing the cell suspension once through an ice-cold Emulsiflex homogenizer set at 17,000 psi.

9. Centrifuge the lysate to remove cell debris (12000 rpm, 30 min, 4°C, Beckman JA12) prior to affinity chromatography.

Affinity chromatography

Note. Perform all subsequent steps at 4°C or on ice.

10. Preequilibrate a 3-ml (bed volume) Glutathione Sepharose column with 20 ml column buffer containing Tween-20.
11. Bind the GST-fusion protein to the column by passing the cleared extract once through the column and save the first flow-through fraction for further processing.
12. Wash the column with 40 ml of column buffer with Tween-20 and then with 20 ml of column buffer without Tween-20.
13. Elute bound protein by applying the elution buffer in 12 × 1 ml increments, and by collecting individual fractions.
14. To increase the total yield of purified GST-fusion protein repeat this affinity chromatography procedure but this time apply the first flow-through fraction to a re-equilibrated column.
15. Determine peak fractions by protein estimation (e.g., with Bradford assay) from both runs, pool the peak fractions, and then desalt into 2 × storage buffer.
16. Measure the absorbance at 280 nm, add an equal volume of glycerol, and store aliquots of the preparation at –20°C.

Notes. The final yields based on the absorbance of the sample in storage buffer (ε=1.032 for a 0.1% (v/v) solution at 280 nm) are of the order of 15 mg/l bacterial culture. While the amount of GST-RNase III for the limited digestion of dsRNA is fixed, we recommend performing a pilot digestion with every new preparation to determine the optimal ratio of dsRNA and GST-RNase III (see Figure 6.3).

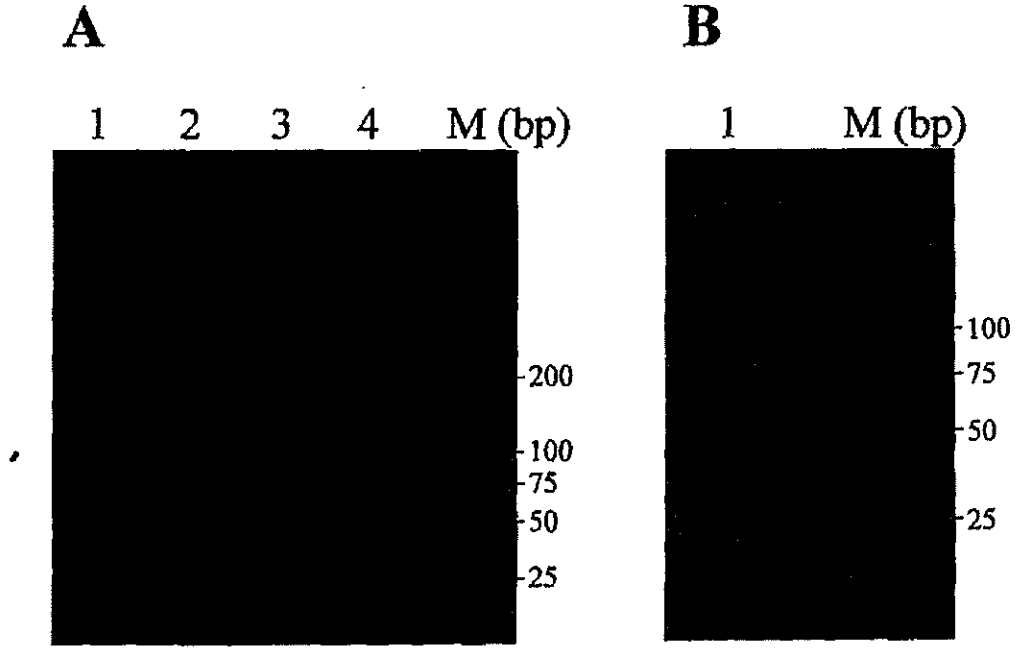

FIGURE 6.3 Titration experiment to determine the optimal amount of RNase III treatment to obtain esiRNA of the correct length. Agarose gels are presented showing the limited digestion of dsRNA before and after purification. Fragment sizes of the ladder are depicted. dsRNA was digested for 4 h with varying amounts of RNase III and stopped by the addition of EDTA. 1.5 µg RNA was loaded per lane. (A) lane 1: dsRNA not digested; lane 2: 40 mg dsRNA digested with 0.75 mg GST-RNase III; lane 3: 40 µg dsRNA digested with 1.5 µg GST-RNase III; lane 4: 40 µg dsRNA digested with 3 µg GST-RNase III. (B) 1.5 µg esiRNA after column purification.

9. Centrifuge the lysate to remove cell debris (12000 rpm, 30 min, 4°C, Beckman JA12) prior to affinity chromatography.

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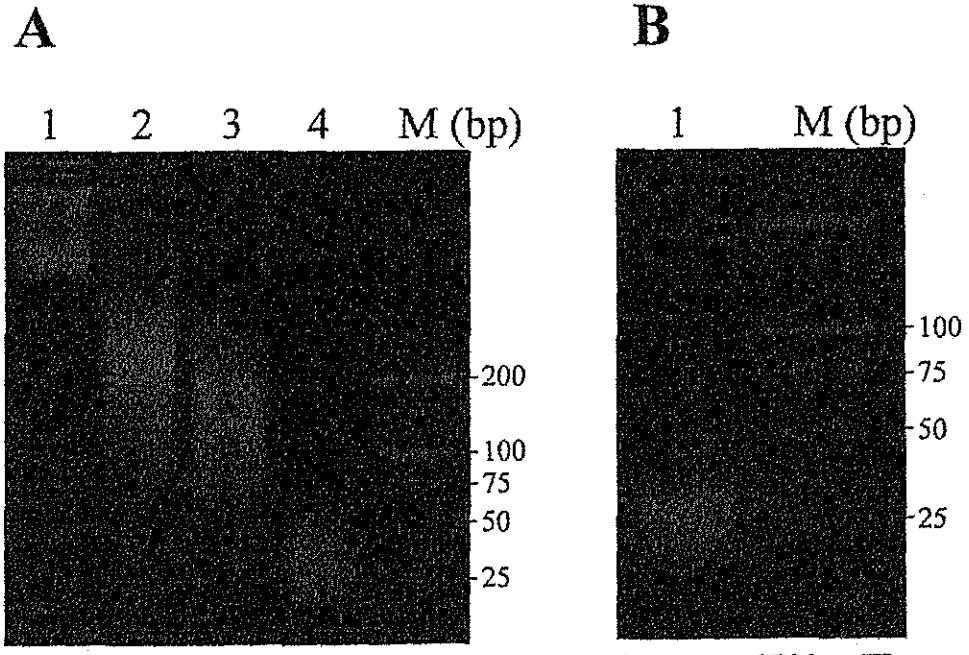

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Protocol 6.3 Generation of PCR Products for *In Vitro* Transcription of Substrate RNAs

The template for *in vitro* transcription can be generated either by amplification of cDNA inserts from clones using primers specific for the vector-backbone or from cDNA preparations using target-specific primers appended with T7 promoter sequences.

When choosing cDNA clones or designing cDNA-specific vectors, it should be taken into account that the amplicon length is critical for the yield of dsRNA. We observed highest yields with amplicon lengths between 400 and 1500 bp. While shorter templates appear to require longer RNase III digestion times, we occasionally observed annealing problems of the two ssRNAs when using templates longer than 1500 bp.

Here we describe the preparation of the template for *in vitro* transcription for the endogenous human gene *Eg5* and the reporter gene *EGFP*. Please note that PCR reaction conditions and cycling parameters depend on the amplicon. Therefore, amplification conditions and buffer composition for other targets may need to be optimized.

Materials

- Taq DNA polymerase and buffers (Bioline)
- Forward and reverse primers with T7 promoter sequence 5'-CGTAATACGACTCACTATAGGG added 5' to a gene-specific or vector-backbone-specific primer sequence; internal T7 promoter sites that are often present in vector backbones can be utilized by choosing a primer to these sites
- Thermal cycler (MJ Research)
- Gene-specific template plasmid

Experimental Procedures

1. Mix the following reagents in a PCR tube:
   - 5 µl 10 × PCR buffer
   - 1.5 µl 50 mM $MgCl_2$
   - 4 µl 10 mM dNTP mix
   - 1 µl 10 µM Forward primer
     5'-CGTAATACGACTCACTATAGGGTGAGCAAGGGCGAGGA (for *EGFP*)
     or
     5'-CGTAATACGACTCACTATAGGGAGAATGTGCTGCAAGGCGATTAAGT (for *Eg5*)
   - 1 µl 10 µM Reverse primer
     5'-CGTAATACGACTCACTATAGGGTACAGCTCGTCCATGCCGA (for *EGFP*) or 5'-TGTGTGGAATTGTGAGCGGATA (for *Eg5*)
   - 2 µl Taq DNA polymerase (1 U/µl)
   - 1–10 ng plasmid template (IMAGE clone 825606 for *Eg5* and for *EGFP*, pEGFPLuc; Clontech or any other plasmid containing the *EGFP* gene)
   - PCR grade water to 50 µl.
2. Amplify as follows: initial denaturation step of 94°C for 3 min; then 40 cycles of 94°C/30 sec, 60°C/30 sec and 72°C/90 sec (*Eg5*) or 72°C/30 sec (*EGFP*); final elongation step of 72°C for 10 min.
3. Analyze products by standard agarose gel electrophoresis.

Protocol 6.4 *In Vitro* Transcription and the Production of dsRNA

PCR products can be used directly for *in vitro* transcription reactions without purification. We routinely use 2.5 µl of the PCR product directly in *in vitro* transcription reactions. The production of dsRNA, including the synthesis of the two RNA strands and their annealing, is performed in a single tube. We have further streamlined this step by skipping the conventional purification steps such as removal of DNA, nucleotides, and precipitation after *in vitro* transcription.

Materials

- MEGAscript™ *in vitro* transcription kit (Ambion)

Experimental Procedures

1. Prepare a 10-µl reaction mix at room temperature as follows:

10 × transcription buffer	1.0 µl
75 mM ATP, GTP, CTP, and UTP	1.0 µl each
PCR product	2.5 µl (~ 0.5 µg DNA template)
Nuclease-free water	1.5 µl
T7 RNA polymerase enzyme mix	1.0 µl

2. Incubate the reaction at 37°C for 4 to 12 h.
3. Perform annealing in a thermal cycler as follows: 90°C for 3 min, ramp to 70°C with 0.1°C/sec, 70°C for 3 min, ramp to 50°C with 0.1°C/sec, 50°C for 3 min, ramp to 25°C with 0.1°C/sec.

Protocol 6.5 Digestion and Purification of dsRNA

Long dsRNA is partially digested to esiRNAs with a length of about 18 to 25 bp. The subsequent purification effectively removes remaining DNA template, unincorporated nucleotides, and dsRNAs longer than 40 bp.

Materials

- dsRNA digestion buffer, pH 7.9 (20 mM Tris-HCl, 0.5 mM EDTA, 5 mM $MgCl^2$, 1 mM DTT, 140 mM NaCl, 2.7 mM KCl, 5% (v/v) glycerol)
- 0.5 M EDTA, pH 8.0
- 4% (v/v) agarose in 1 × TBE electrophoresis buffer
- 25 bp DNA ladder (e.g., HyperLadder V from Bioline)
- TE buffer, pH 7.9 (10 mM Tris-HCl, 1 mM EDTA)
- Isopropanol
- 70% (v/v) ethanol
- Micro Bio-Spin Chromatography Columns (BioRad Laboratories)
- Q Sepharose FastFlow (Amersham Biosciences)
- Equilibration buffer (20 mM Tris, 1 mM EDTA, 300 mM NaCl, pH 8.0)
- Wash buffer (20 mM Tris, 1 mM EDTA, 400 mM NaCl, pH 8.0)
- Elution buffer (20 mM Tris, 1 mM EDTA, 520 mM NaCl, pH 8.0)

- Ethidium bromide, 10 mg/ml (Sigma-Aldrich)
- Orange G (Sigma-Aldrich); marker dye for esiRNAs

Experimental Procedures

1. Mix 10 µl of the *in vitro* transcription reaction (~25-50 µg dsRNA) with 3 µg of GST-RNase III protein in 90 µl digestion buffer (see also Figure 6.3).
2. Incubate at 23°C for 4 h.
3. Run a 2–4 µl aliquot in 4% (w/v) agarose gel along with a 25-bp DNA ladder to check the size range of digestion products. If the size range is not appropriate (i.e., digestion products are too long), incubate for additional 2 h at 37°C and check again.
4. Purify the digestion products immediately as follows, or terminate the reaction by adding 4 µl 0.5 M EDTA.
5. Prepare spin columns for purification. Add 200 µl Q-Sepharose and 500 µl equilibration buffer to the column. Spin at 1000 g for 1 min and discard the flow-through.
6. Again, add 500 µl equilibration buffer, spin at 1000 g for 1 min, and discard the flow-through.
7. Load all of the digested dsRNA onto the column and incubate for 5 min at room temperature.
8. Spin at 1000 g for 1 min and discard the flow-through.
9. Add 500 µl wash buffer, spin at 1000 g for 1 min, and discard the flow-through.
10. Add 300 µl elution buffer, spin at 1000 g for 1 min, and **collect** the flow-through.
11. Repeat step 10.
12. Add 500 µl isopropanol to the 600 µl eluted esiRNA and vortex. Store on ice for at least for 30 min.
13. Spin at 16,000 g for 15 min at 4°C. Discard the supernatant and wash the pellet twice with cold 70% (v/v) ethanol.
14. Air-dry the esiRNA pellet for 10 to 15 min at room temperature and dissolve in 50 µl of 1 × TE buffer.
15. Run a 2–4 µl aliquot on a 4% (w/v) agarose gel, and measure OD_{260} in order to quantify the esiRNA concentration.

Protocol 6.6 Transfection of esiRNA and Analysis of Silencing in Cultured Mammalian Cells

Optimal transfection conditions for esiRNA are highly dependent on the cell line used. Here we describe the silencing of the endogenous gene *Eg5* in HeLa cells (Figure 6.4). The knockdown of *Eg5* results in a mitotic arrest phenotype within 24 hours after transfection and is, therefore, suitable for optimizing transfection conditions in human cell lines.[22] We also describe inhibition of the reporter gene *EGFP*. These two genes can serve as the template for designing other esiRNAs directed against specific genes of interest. However, also consider the following when designing esiRNAs against specific genes. First, each esiRNA directed against a specific gene may have a different silencing capacity. This silencing capacity can be evaluated by quantitative RT-PCR,

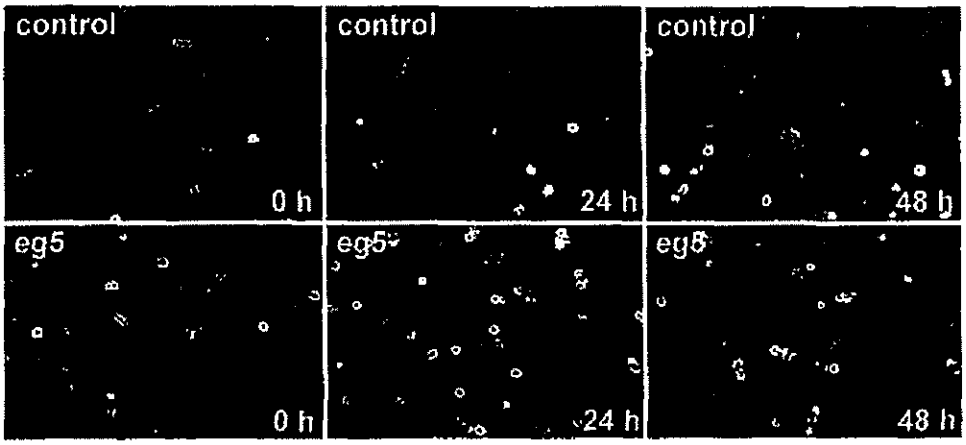

FIGURE 6.4 Transfection of esiRNA into mammalian cells and phenotypic analysis. The upper panel (control) shows HeLa cells transfected with 0.5 μg of luciferase esiRNA photographed at indicated time points. The lower panel (eg5) shows HeLa cells transfected with 0.5 μg esiRNA directed against the kinesin eg5 at the same time points.

northern blot or western blot analyses. Second, each gene product has a different half-life within the cell. Therefore, some phenotypes may be observed earlier then others. Hence, cells should be examined at different time points post-esiRNA transfection. Third, RNAi leads to gene knockdown and not knockout. Therefore, some gene product may still be present in the cell at sufficient concentrations to fulfill its function.

The experiments described here are for transfections in 6-well dishes. Adjust volumes and amount of reagents for tissue culture dishes of a different size.

Materials

- Oligofectamine and Lipofectamine 2000 (Invitrogen); other transfection reagents and protocols should work accordingly
- Dulbecco's Mod Eagle Medium (D-MEM) (InVitrogen)
- Fetal bovine serum (FBS) (InVitrogen)
- Other tissue culture ingredients; L-Glutamine, penicillin/streptomycin (InVitrogen, Sigma-Aldrich)

Experimental Procedures

Knockdown of an endogenous gene (Eg5)

1. Plate cells ~16 h before transfection in a 6-well culture dish to obtain 30 to 50% confluency on the day of transfection.
2. Change the medium (10% (v/v) FBS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, DMEM) before transfection. The volume of the medium for transfection should be 800 μl.
3. Dilute 0.5 μg of esiRNA directed against *Eg5* with 185 μl of serum-free DMEM.
4. Dilute 4 μl of Oligofectamine with 11 μl of serum-free medium and incubate at RT for 5 min.
5. Add diluted esiRNA to diluted Oligofectamine. Incubate at RT for 20 min.
6. Add esiRNA/Oligofectamine mix to the cells. Gently mix and incubate at 37°C for 4 h.

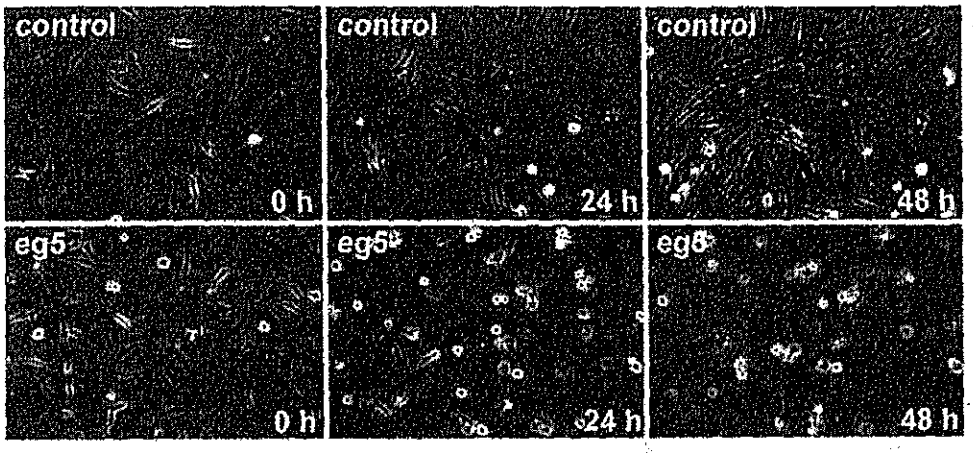

FIGURE 6.4 Transfection of esiRNA into mammalian cells and phenotypic analysis. The upper panel (control) shows HeLa cells transfected with 0.5 μg of luciferase esiRNA photographed at indicated time points. The lower panel (eg5) shows HeLa cells transfected with 0.5 μg esiRNA directed against the kinesin eg5 at the same time points.

northern blot or western blot analyses. Second, each gene product has a different half-life within the cell. Therefore, some phenotypes may be observed earlier then others. Hence, cells should be examined at different time points post-esiRNA transfection. Third, RNAi leads to gene knockdown and not knockout. Therefore, some gene product may still be present in the cell at sufficient concentrations to fulfill its function.

The experiments described here are for transfections in 6-well dishes. Adjust volumes and amount of reagents for tissue culture dishes of a different size.

Materials

- Oligofectamine and Lipofectamine 2000 (Invitrogen); other transfection reagents and protocols should work accordingly
- Dulbecco's Mod Eagle Medium (D-MEM) (InVitrogen)
- Fetal bovine serum (FBS) (InVitrogen)
- Other tissue culture ingredients; L-Glutamine, penicillin/streptomycin (InVitrogen, Sigma-Aldrich)

Experimental Procedures

Knockdown of an endogenous gene (Eg5)

1. Plate cells ~16 h before transfection in a 6-well culture dish to obtain 30 to 50% confluency on the day of transfection.
2. Change the medium (10% (v/v) FBS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, DMEM) before transfection. The volume of the medium for transfection should be 800 μl.
3. Dilute 0.5 μg of esiRNA directed against *Eg5* with 185 μl of serum-free DMEM.
4. Dilute 4 μl of Oligofectamine with 11 μl of serum-free medium and incubate at RT for 5 min.
5. Add diluted esiRNA to diluted Oligofectamine. Incubate at RT for 20 min.
6. Add esiRNA/Oligofectamine mix to the cells. Gently mix and incubate at 37°C for 4 h.

7. Add 1000 µl serum-containing medium.
8. Observe knockdown of *Eg5* by light microscopy within 24 h posttransfection. At this time, many of the cells should be arrested in mitosis, indicated by the rounded-up appearance. Most of the cells should die within 48 hours posttransfection (see also Figure 6.4).

Knockdown of EGFP expressed from a plasmid

1. Plate cells ~16 h before transfection into a 6-well culture dish to achieve 80 to 90% confluency on the day of transfection.
2. Change the medium (10% (v/v) FBS, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, DMEM) before transfection. The volume of the medium for transfection should be 2000 µl.
3. Dilute 1 µg of esiRNA directed against *EGFP* or a control esiRNA (e.g., firefly luciferase) and 3 µg of EGFP expression plasmid with 250 µl of serum-free DMEM.
4. Dilute 10 µl of Lipofectamine 2000 in 250 µl of serum-free medium, and incubate at RT for 5 min.
5. Add diluted esiRNA-plasmid to diluted Lipofectamine 2000. Incubate at RT for 20 min.
6. Add esiRNA-plasmid/Lipofectamine 2000 mix to the cells. Gently mix and incubate at 37°C for 4 hours.
7. Remove old medium and add 2500 µl serum-containing medium.
8. Observe the cultures using appropriate filter sets for GFP visualization 24 h post-transfection. One should see a clear fluorescence intensity difference between cells transfected with control esiRNA and EGFP esiRNA.

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