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Silencing Genes by RNA Interference in the Protozoan Parasite *Entamoeba histolytica*

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Summary

Experimental procedures using the RNA interference (RNAi) approach have recently emerged as a powerful tool for gene silencing in eukaryotic microbes for which gene replacement techniques have not yet been developed. Our group has recently explored RNAi to knock down gene-specific expression in the protozoan parasite *Entamoeba histolytica*, through delivery of small interfering RNA (siRNA) oligonucleotides by the soaking approach.

Standardized conditions for the soaking of *E. histolytica* trophozoites with siRNAs result in highly specific and significant silencing of parasite cognate genes. Real-time PCR analysis indicates that a 16-hour treatment with siRNAs usually results in half-extinction of target messenger RNA. Furthermore, Western blot analysis of trophozoite crude extracts with the use of specific antibodies shows a similar reduction of cognate protein levels after siRNA treatment.

Key Words: *Entamoeba histolytica;* gene silencing; RNA interference (RNAi); small interfering RNA (siRNA).

1. Introduction

The protozoan parasite *Entamoeba histolytica* is the etiological agent of amoebiasis, which is the third leading cause of death from parasitic disease worldwide ***(1)***. Around 50 million cases of invasive amoebiasis are declared annually, resulting in nearly 100,000 deaths.

In silico analysis of *E. histolytica*-annotated genome predicts around 8,343 genes ***(2)*** (*E. histolytica* genome project; Web site http://www.tigr.org/). However, the role of a small percentage of these genes, especially in the host–parasite relationship, is currently known. A strong effort has been made in the

From: *Methods in Molecular Biology, vol. 442: RNAi: Design and Application*
Edited by: S. Barik © Humana Press, Totowa, NJ

field of amoebiasis to develop molecular tools capable of knocking down gene-specific expression. Most of the studies that aim to characterize a particular *E. histolytica* phenotype rely on the use of antisense strategies ***(3)***. However, this approach requires transformation of trophozoites (vegetative stage of the parasite) with plasmid constructs, maintained episomally by antibiotic selection at uncertain copy numbers. Additionally, plasmid constructs engineered for the expression of antisense messenger RNA are under the control *E. histolytica* transcriptional elements, which are still poorly understood.

RNAi has recently emerged as a powerful methodology to knock down gene expression in a variety of eukaryotic organisms, including *Entamoeba histolytica* ***(4)***. Some of the key enzymes participating in the RNAi pathway have recently been identified throughout the *E. histolytica*-annotated genome ***(5)***. The RNAi pathway is triggered by long double-stranded RNA that is processed by an enzyme called "Dicer" into small interfering RNAs (siRNAs). Resulting siRNAs assemble into endoribonuclease-containing complexes known as RNA-induced silencing complexes (RISCs), unwinding in the process. The siRNA strands subsequently guide the RISC to complementary RNA molecules, where they cleave and destroy the cognate RNA. Under experimental conditions, chemically synthesized siRNAs can be used to induce specific messenger RNA degradation (soaking approach) for eukaryotic cells cultured *in vitro*.

This chapter presents standard methods to knock down gene expression of *E. histolytica* trophozoites cultured *in vitro* by using the soaking approach with siRNAs (**Fig. 1**) ***(6)***. The efficiency of siRNA soaking is evaluated through real-time PCR to quantify messenger RNA extinction. Standard Western blotting procedures of trophozoite crude extracts to verify protein reduction are also described. Finally, we should mention that although RNAi technology can produce a null-phenocopy for some genes, it is by no means a substitute for genetic mutants.

2. Materials

2.1. Preparation of siRNAs

1. siRNA: A set of chemically synthesized (sense and antisense) siRNA oligonucleotides per target gene can be purchased from Eurogentec (Liège, Belgium; http://www.eurogentec.com) or other specialized companies. As a negative control, an additional set of sense and antisense primers can be made with either a scrambled sequence of the siRNA of interest (this control allows us to check the specificity of the siRNA of interest) or a perfectly unique sequence that should not match any sequence in the genome of *E. histolytica*. For the second kind of negative control, we recommend using an siRNA

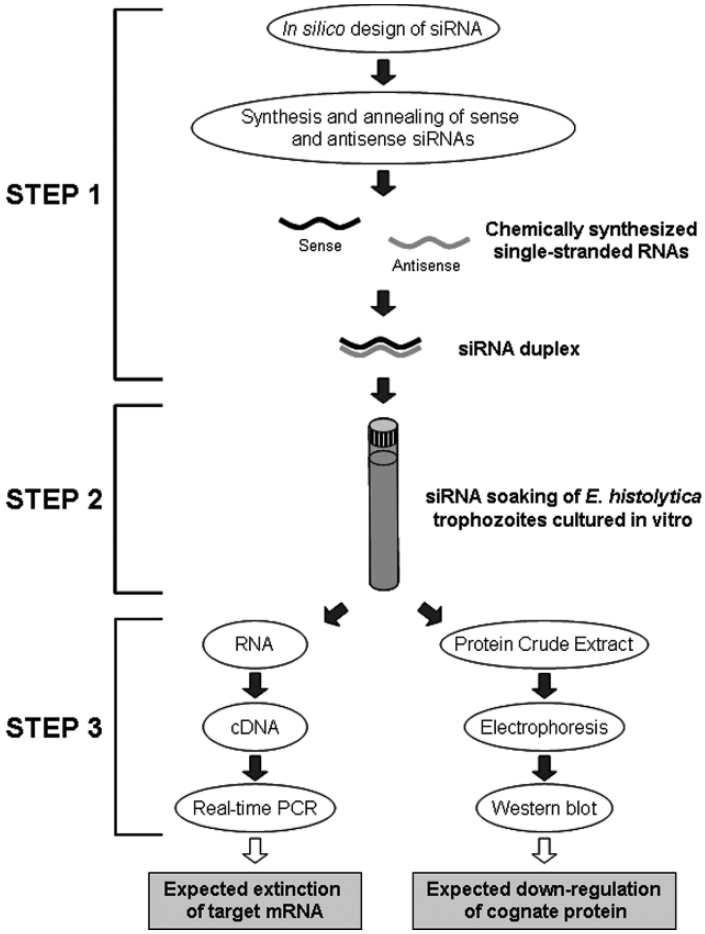

Fig. 1. Schematic overview of standard procedures followed to set up an *E. histolytica* siRNA soaking experiment. A set of sense and antisense RNA oligonucleotides is designed *in silico* based on target gene sequence (step 1). Sense and antisense RNA oligonucleotides are annealed to form the siRNA duplex that will be used to induce specific gene silencing in *E. histolytica* trophozoites cultured *in vitro* (step 2). After the soaking with siRNA, the phenotype of trophozoites is characterized to verify target mRNA extinction and cognate protein downregulation. Trophozoite RNA is purified to synthesize target and endogenous cDNA sequences that will be used as templates to quantify through real-time PCR the expected extinction of target mRNA. After the soaking with siRNA, trophozoite crude extract is also prepared to verify through Western blot the expected downregulation of cognate protein (step 3).

duplex (5′-CAAGCUGACCCUGAAGUUCdTdT for the sense strand and 5′-GAACUUCAGGGUCAGCUUGdTdT for the antisense strand) based on the green fluorescence protein (GFP) coding sequence, which is nonexistent in *E. histolytica* (*see* **Note 1**).
2. Annealing buffer (5X): 50 m*M* Tris HCI, pH 7.5–8.0, 100 m*M* NaCl in RNase-free water.
3. Nuclease-free water (Ambion, Austin, TX).
4. RNase-free microcentrifuge tubes and a water bath at 95 °C.

2.2. Trophozoite Culture and Crude Extract Preparation

1. HM1:IMSS *E. histolytica* strain: We use culture procedures previously described by Diamond et al. *(7)*. Briefly, *E. histolytica* trophozoites are maintained under axenic conditions through successive passages from culture tubes placed horizontally in an incubator at 37 °C until ~90% confluence (~1×10^6 trophozoites/tube). Once confluence is reached, culture media supernatant is carefully decanted and adherent cells are rinsed gently with 2 mL per tube of TYI-S-33 medium previously incubated at 37 °C. Tubes are immediately refilled with 5 mL of fresh medium, and trophozoites are detached by striking the bottom of tubes three times against the workbench surface. The cellular suspension that is obtained is used immediately to prepare new culture tubes.
2. Trypticase yeast extract, iron, and serum medium (TYI-S-33) is prepared using the following procedure: A mixture containing 3% (w/v) biosate peptone, 50.5 m*M* glucose, 5.7 m*M* L-cysteine, 1 m*M* (+)-sodium L-ascorbate, 34.2 m*M* NaCl, 5.7 m*M* K_2HPO_4, 4.4 m*M* KH_2PO_4, 90 μ*M* ferric ammonium citrate, in deionized water, is autoclaved for 15 min at 110 °C. The autoclaved mixture is cooled overnight at 4 °C before use. Alternatively, the cooled mixture can be stored at –20 °C before use for at least 3 months (the frozen mixture is defrosted in a water bath at 37 °C before use). Fresh TYI-S-33 medium is prepared with autoclaved cooled mixture supplemented with 15% (v/v) heat-inactivated adult bovine serum (PPA Laboratories, Linz, Austria) (serum is inactivated 40 min in a water bath at 55 °C), 3% (v/v) Diamond Vitamin Tween 80 solution (40X) (JRH Biosciences, Lenexa, KS), 100 U/mL of penicillin, and 100 μg/mL of streptomycin (Gibco-BRL, Bethesda, MD). TYI-S-33 medium is filter-sterilized with a 0.2-μm vacuum filtration system (e.g., Nalgene, TTP LabTech, etc.) and stabilized at 37 °C in a water bath for at least 30 min before use. TYI-S-33 medium can be stored at 4 °C no longer than 24 h.
3. Nunclon™Δ polystyrene culture tubes of 8-mL capacity with screw caps (Nalge Nunc, Naperville, IL).
4. Standard cell culture incubator with a rack to place culture tubes horizontally and water-bath-stabilized at 37 °C.
5. Sterile phosphate buffer saline (PBS): 137 m*M* NaCl, 2.7 m*M* KCl, 10 m*M* Na_2HPO_4, 1.8 m*M* KH_2PO_4, pH 7.4, with HCl.
6. Cell lysis buffer (2X): One tablet of complete, Mini, EDTA-free, protease inhibitor cocktail (Roche, Mannheim, Germany) is dissolved in 3.5 mL of 10 m*M* Tris-HCl

pH 7.5, supplemented with 10 μ*M* of leupeptin, 1 m*M* of NEM, 2 m*M* of PHMB, 2 m*M* of AEBSF, and stored in single-use aliquots at –20 ºC. Working solution is prepared by dilution in 10 m*M* Tris-HCl, pH 7.5, and 10% (w/v) SDS.

2.3. Total RNA Extraction

1. Workbench surface and automatic micropipettes are wiped with a nuclease decontaminant reagent (e.g., RNase-away from Ambion, Invitrogen, etc.).
2. RNase-free microcentrifuge tubes and micropipette tips.
3. TRIzol™ reagent (Invitrogen, Carlsbad, CA).
4. RNase-free water prepared by treating water overnight with 0.01% (v/v) of diethylpyrocarbonate (DEPC) into RNase-free glass bottles and autoclaving.
5. Chloroform, isopropanol, and 75% ethanol prepared with RNase-free water.
6. Spectrophotometer with 260- and 280-nm filters, and suitable quartz measuring cells.
7. Standard horizontal electrophoresis unit.
8. Electrophoretic-grade agarose powder (e.g., Invitrogen, Bio-Rad) and Tris-Borate-EDTA (TBE) buffer (89 m*M* Tris, 89 m*M* boric acid, pH 8.3, in RNase-free water).
9. Sample buffer (10X): 35% (v/v) glycerol, 10 m*M* of EDTA, 0.25% (w/v) xylene cyanol, 0.25% (w/v) bromophenol blue, in RNase-free water.
10. Ethidium bromide solution: 0.2 mg/mL in RNase-free water. Ethidium bromide is a potent mutagen that should be handled with care.
11. 1 kb DNA Ladder (New England Biolabs, Ipswich, MA).

2.4. First-Strand cDNA Synthesis (Reverse Transcription)

1. Total RNA samples diluted in nuclease-free water to a final concentration of 0.6–1 μg/μL (each reverse-transcription reaction is set with 3–5 μg).
2. Reverse-transcription primers (about 20–22 nucleotides, usually close to stop codon to amplify most of the gene) based on complementary strand sequence for target gene and another endogenous control gene that will be used as a normalizer. For endogenous genes we suggest the glyceraldehyde-3-phosphate dehydrogenase (G3PDH: GenBank accession no. M89790) or the ribosomal protein L9 (four copies of the gene, GenBank accession nos. 117.t00010, 117.t00014, 312.t00001, 465.t00004). The following primers have been successfully tested for reverse-transcription reaction: G3PDH: 5′-CTAGCTGGGATAATGTTAG and L9: 5′-TGATGCAATAGTTCTAAC.
3. Reverse-transcription primer mix (RT-Mix) containing one reverse primer for the target gene and one reverse primer for the endogenous control gene (0.3 μ*M* each in nuclease-free water).
4. dNTP mix (10 m*M* each) from Invitrogen or other specialized company.
5. Reverse transcriptase SuperScript™ II RNase H– (Invitrogen).
6. Ribonuclease inhibitor RNasin (Promega, Madison, WI) (40 U/μL).
7. Water baths at 42 °C and 70 °C.

2.5. Real-Time PCR

1. Two sets of forward and reverse primers (about 20–22 nucleotides in length each and upstream from reverse-transcription primer), to amplify target sequence and endogenous control sequence (~100 bases), with melting temperatures close to 55 °C. As an endogenous control sequence for *E. histolytica*, we suggest a set of primers based on either G3PDH (5′-GTTAATGGACACAAGATTG and 5′-TTCAACAACATAATCAACTC) or ribosomal protein L9 (5′-CTTGTTGTAA-AGAAGGAAGGAC and 5′-AACAGCTGAATCTCTTCTATTTC) genes.
2. Nuclease-free water (Ambion).
3. SYBR™ Green PCR Master Mix (Applied Biosystems, Foster City, CA) (this reagent contains potential carcinogens and should be handled with care).
4. Repetition micropipette capable of dispensing 5–15 µL each time and suitable nuclease-free tips.
5. Optical 96- or 384-well reaction plates, optical adhesive covers, and MicroAmp™ adhesive film applicator (Applied Biosystems).
6. Real-time quantitative PCR system compatible with 96- or 384-well plates (e.g., Applied Biosystems' 7900HT Fast Real-Time PCR).

2.6. SDS-PAGE

1. Standard vertical SDS-PAGE system to set up 1.5-mm-thick minigels [e.g., Mini-Protean system (Bio-Rad, Hercules, CA) or Mighty Small SE250/260 (Hoeffer, San Francisco, CA)].
2. Separating buffer (4X): 1.5 *M* Tris-HCl, 0.4% (w/v) SDS. Adjust pH to 8.8 and store at room temperature.
3. Stacking buffer (4X): 0.5 *M* Tris-HCl, 0.4% (w/v) SDS. Adjust pH to 6.8 and store at room temperature.
4. Thirty percent acrylamide-bisacrylamide solution (29:1 ratio) (neurotoxic reagent that should be handled with care) and tetramethylethylenediamine (TEMED) (Euromedex, Souffelweyersheim, France).
5. Freshly prepared 10% (w/v) ammonium persulfate solution.
6. Isopropanol.
7. Running buffer: 25 m*M* Tris-base (do not adjust pH), 192 m*M* glycine, 0.1% (w/v) SDS. The final pH will be around 8.3. Store at room temperature.
8. Sample treatment buffer (2X): 0.125 *M* Tris-HCl, 4% (w/v) SDS, 20% (v/v) glycerol, 2% (v/v) ß-mercaptoethanol, 0.002% (w/v) bromophenol blue, pH 6.8. Store single-use aliquots at –20 °C.
9. Prestained molecular weight markers: Kaleidoscope markers (Bio-Rad).

2.7. Western Blot to Check Target Protein Level

1. Mini Trans-Blot Electrophoretic Transfer Cell from BioRad or standard minigel transfer unit.
2. Transfer buffer: 50 m*M* Tris-base, 50 m*M* boric acid. Store at room temperature.

3. Immobilon PVDF sequencing membrane (Millipore, Bedford, MA), and 3MM Chr filter paper (Whatman, Maidstone, UK).
4. Methanol.
5. Washing buffer: PBS with 0.05% (v/v) of Tween-20.
6. Blocking buffer: 5% (w/v) nonfat dry milk in washing buffer.
7. Primary antibody diluted in blocking buffer.
8. Secondary antibody: Usually anti-mouse or anti-rabbit IgG antibody conjugated to horseradish peroxidase (Jackson Laboratories, West Grove, PA), diluted in washing buffer.
9. Enhanced chemiluminescent (ECL)-Plus reagents (Amersham Biosciences, Buckinghamshire, UK), BioMax MR film (Kodak, Rochester, NY), and automatic developing device.

2.8. Stripping and Reprobing Blots

1. Stripping buffer: 100 m*M* ß-mercaptoethanol, 2% (w/v) SDS, and 62.5 m*M* Tris-HCl, pH 6.7. Store at room temperature.
2. Washing buffer and blocking buffer as in previous section.

3. Methods

The first step to set up siRNA-soaking experiments is to purchase chemically synthesized single-stranded siRNA oligonucleotides based on partial or complete coding sequence of a target gene. Several companies offer standard automated procedures for the design and synthesis of highly efficient single-stranded siRNA oligonucleotides. Briefly, *in silico* design of siRNA sequences that are 19 nucleotides (nt) in length is performed by using a series of algorithms based on most documented features relevant for effective RNAi ***(8)***. Users select a 19-nt sequence among best candidates (companies usually propose three to five free siRNA designs) to order the synthesis of two complementary sense and antisense RNA oligonucleotides. A two-nucleotide 3′ overhang (either UU or dTdT di-nucleotides) is added to each RNA oligonucleotide to ensure that the sequence-specific endonuclease complexes are efficiently formed. In general, the best 21-nt siRNA sequence is selected using the following guidelines:

1. Among the siRNA designs, select the 21-nt sequence with GC content closest to 50%.
2. Verify that the 21-nt sequence has no long base runs (e.g., TTTT or CCCC).
3. Check that the selected sequence targets only one gene [21-nt sequence is subjected to a BLAST-search (NCBI database) against the *E. histolytica* genome].

After treatment with siRNAs, reliable characterization of the *E. histolytica* phenotype depends on successful messenger RNA degradation. Therefore, it

is important to quantify the amount of target mRNA for experimental siRNA-treated trophozoites, compared to nonspecific siRNA-treated trophozoites and wild-type trophozoites. A quantitative real-time PCR approach is used to determine the relative amount of mRNA for each condition. An endogenous gene (e.g., *G3PDH*) is used to normalize relative quantification. It is expected that partial degradation of cognate mRNA will decrease the amount of translated protein. Reduction in the level of cognate protein is verified through Western blot analysis of *Entamoeba* crude extracts by using specific antibodies. Another endogenous protein (usually a housekeeping product; e.g., actin, tubulin, etc.) is used as control to normalize the blots.

3.1. Annealing of siRNA Oligonucleotides

1. Spin down the tubes containing the dried RNA oligonucleotide at 13,200g for 1 min. Resuspend each oligonucleotide at a concentration of 50 μ*M* (~1.4 μg/μL), in nuclease-free water, and store at –20 °C before use (*see* **Note 1**).
2. For each siRNA duplex (i.e., specific siRNA duplex and control-siRNA duplex), mix 30 μL of each sense and antisense oligonucleotide solution with 15 μL of 5X annealing buffer (50 m*M* of Tris, pH 7.5–8.0, 100 m*M* of NaCl in RNase-free water). The annealing mix (final volume: 75 μL) has an siRNA duplex concentration of 20 μ*M* (~1.1 μg/μL).
3. Incubate annealing mix for 2 min at 95 °C in a water bath and allow to cool at room temperature for 30–45 min. Spin down the tubes as before to collect all liquid at the bottom of the tube, and keep the tubes on ice until use.

3.2. Quantification of siRNA Duplex

1. Prepare a 1:200 dilution of the annealed siRNA duplex in deionized water (e.g., 2 μL of RNA and 398 μL of water).
2. Set the spectrophotometer wavelengths to 260 and 280 nm, and blank with deionized water. Measure the optical density (O.D.) of diluted RNA.
3. Determine RNA concentration (μg/μL) by using the following equation (an O.D. of 1 at 260 nm corresponds to 40 μg/mL of RNA): (45 × O.D. at 260 nm × dilution factor)/1000. Ideally, the ratio between readings at 260 and 280 nm is at least 2.0 for pure preparations of RNA.

3.3. Soaking of E. histolytica Trophozoites with siRNA

1. Prepare a trophozoite suspension by using a ~90% confluent culture as described above (see Section 2 under HM1:IMSS *E. histolytica* strain). Determine the cellular density of the trophozoite suspension by counting the number of cells with a Malassez chamber.
2. Fill culture tubes with 8 mL of TYI-S-33 previously incubated at 37 °C (at least two identical tubes per condition: one to purify total RNA and the other to prepare

trophozoite crude extract). Inoculate each tube with 1×10^5 trophozoites and culture horizontally at 37 °C until ~50% confluence is reached (~24 h later).
3. Add 0.8 mg/tube of annealed siRNAs (i.e., 10 µg of siRNA duplex per mL of culture media) and culture overnight (~16 h) before the analysis of phenotype (*see* **Note 2**).

3.4. Total RNA Purification

1. Decant culture media supernatant from each tube (i.e., wild-type, nonspecific siRNA duplex and gene-specific siRNA duplex), and gently rinse adherent trophozoites once with 2 mL of PBS previously incubated at 37 °C.
2. Discard PBS rinse and immediately add 1 mL of TRIzol to each culture tube. Mix several times by inverting the tubes until trophozoites are completely detached, and incubate for 5 min at room temperature.
3. Transfer separately lysis reactions to 2-mL microcentrifuge tubes. Add 0.2 mL of ice-cold chloroform to each tube, and mix by inverting 4–6 times. Centrifuge at 9,500g for 15 min at 4 °C.
4. Following centrifugation, carefully collect the upper colorless phase with a micropipette, and transfer to fresh 1.5-mL microcentrifuge tubes. Avoid contamination with white interphase and lower phenol/chloroform red phase, since this could reduce the purity of RNA samples.
5. Add 0.5 mL of ice-cold isopropanol to each tube containing upper colorless phase, and incubate for 30 min at – 20 °C before centrifugation at 13,200g for 15 min at 4 °C.
6. Wash RNA pellet once with 1 mL of ice-cold 75% ethanol and centrifuge for 5 min as before.
7. Briefly air-dry RNA pellet for 5–10 min and dissolve in nuclease-free water (20–30 µL per culture tube) by incubating for 5 min at 55 °C and pipetting a few times. Alternatively, dry RNA pellets can be stored at –70 °C. Before using dissolved RNA, quantify samples following the same procedure as for annealed siRNA quantification (*see* **Section 3.2** under *Quantification of siRNA duplex*).

3.5. Electrophoretic Analysis of RNA Samples

1. One percent (w/v) agarose gel is prepared in TBE buffer. Mix 1 g of agarose powder with 100 mL of TBE buffer (enough volume to fill standard tray with a surface of 80 cm^2) in a 500-mL Erlenmayer flask. Melt the agarose in a microwave at medium temperature for approximately 5 min or more with occasional shaking. Alternatively, place the Erlenmayer flask on a heating plate with magnetic stirrer (boiling temperature is required for proper melting of agarose; therefore, the use of safety goggles and gloves is recommended). Melted agarose is cooled down to approx. 50–60 °C at room temperature. Pour agarose into a casting tray containing a thin comb (~1 mm). When the gel has solidified, remove the comb and place the casting tray in electrophoresis tank. Fill electrophoresis tank with TBE-buffer approximately 1 cm over gel surface.

2. Prepare each sample by mixing 8 µL of RNA previously diluted in RNAse-free water (~1 µg) with 1 µL of sample buffer and 1 µL of ethidium bromide solution.
3. Load molecular marker (~1 µg/lane) and RNA samples, previously diluted in loading buffer into TBE–agarose gel wells by using a micropipette.
4. Place the tank lid and connect electrical leads to power supply with proper orientation (anode to anode in red, and cathode to cathode in black) and in the same channel. Turn on electrophoresis power supply and set voltage to 100. Electrophoresis is accomplished at 100 V for 30–45 min.
5. Visualize RNA bands by placing the gel on an UV transilluminator (UV radiation can be dangerous for unprotected eyes and skin; therefore, UV protective goggles or a face shield must be worn). Typically, ethidium bromide staining of total RNA shows two bands comprising the 28S and 18S ribosomal RNA species and other bands where smaller RNA species are located. RNA is considered of high quality when the 28S band is approximately two times more intense than the 18S band.

3.6. Synthesis of cDNA by Reverse-Transcription (RT)-PCR

1. For each condition (i.e., wild-type trophozoites and trophozoites treated with either nonspecific siRNA or specific siRNA), mix in a microcentrifuge tube 5 µL of total RNA (3–5 µg) with 6 µL of RT-mix and 1 µL of dNTP mix.
2. Incubate tubes for 3 min at 70 °C and immediately place on ice. Add 4 µL of first-strand 5X buffer, 2 µL of DTT, 1 µL of RNasin, and 1 µL of reverse transcriptase. Incubate reaction mix for 50 min at 42 °C (annealing step) and immediately place tubes at 70 °C for 15 min (extension step). Synthesized cDNA can now be used as a template for real-time PCR or stored at –20 °C until use (*see* **Note 3**).

3.7. Quantitative Real-Time PCR

1. Dilutions of a cDNA sample prepared from wild-type *E. histolytica* trophozoite total RNA are used to construct standard curves for the target gene and the endogenous gene (i.e., *G3PDH*) amplifications. Prepare by triplicate five 10-fold serial dilutions of wild-type *E. histolytica* cDNA sample using nuclease-free water and microcentrifuge tubes (dilutions of 1:10, 1:100, 1:1000, 1:10,000, and 1:100,000 usually result in reliable data). To prepare the first dilution (i.e., 1:10), mix 10 µL of cDNA sample and 90 µL of water. Collect liquid at the bottom of the tube by briefly centrifuging for 30 sec at 13,200g. Each subsequent dilution is a 10-fold dilution made from a previous dilution (e.g., dilution 1:100 = 10 µL of dilution 1:10 + 90 µL of water).
2. The unknown samples correspond to cDNA prepared from total RNA isolated from trophozoites treated with either specific siRNA or nonspecific siRNA (control). For each unknown sample, prepare three serial dilutions by triplicate as above (dilutions of 1:50, 1:500, and 1:5,000 of *Entamoeba histolytica* cDNA samples usually work as a template for real-time PCR). For the initial dilution (i.e., 1:50), mix 2 µL of RT-PCR and 98 µL of nuclease-free water by vortexing.

Each subsequent dilution is a 10-fold dilution made from the previous dilution as described above (e.g., dilution 1:500 = 10 µL of dilution 1:50 + 90 µL of water).

3. Separately prepare two real-time PCR mixes (a mix for target gene and a mix for another endogenous gene that will be used as a normalizer) in 2-mL microcentrifuge tubes, by mixing 20 µL of each forward and reverse primer (stock: 100 µM each), 460 µL of nuclease-free water, and 1 mL of SYBR™ Green PCR Master Mix (final volume of 1.5 mL per mix, enough to set 96 amplifications).
4. Set up real-time PCR using either 96 or 384 microtiter plates in order to have two identical triplicates for each cDNA dilution including both standard curves and unknown samples (i.e., amplification template). Each amplification reaction is prepared directly in microtiter plate wells by mixing 5 µL of cDNA per well with 15 µL of either target gene real-time PCR mix or endogenous gene real-time PCR mix (i.e., 20 µL per amplification/well). The use of an automatic repetition micropipette is recommended to prepare the amplification reactions.
5. Plates are sealed with an optical adhesive cover by applying pressure all along the surface with a rubber applicator (alternatively, use fingertips and soft tissue paper). Sealed plates are briefly centrifuged for 5 min at 300g and kept at 4 °C while the PCR machine is programmed.
6. Carry out PCR using SYBR Green as detector, ROX as passive reference, and the following thermal cycling parameters: (a) initial step, 10 min at 95 °C (hot start); (b) 35 cycles: 15 sec at 95 °C (denaturation), 30 sec at 55 °C (annealing), and 15 sec at 72 °C (extension).
7. The amount of target mRNA for each experimental condition is calculated using the relative standard curve method described in Applied Biosystems' user bulletin #2 (Relative quantitation of gene expression, ABI PRISM 7700 Sequence Detection System; Product bulletin # 4303859: Web site: http://www.appliedbiosystems.com/). Briefly, the relative amount of target and endogenous mRNA for each experimental sample is determined using the appropriate standard curve. Then the target amount is divided by the endogenous reference amount to obtain a normalized target value. Each of the normalized target values is divided by the calibrator-normalized value (i.e., control trophozoites treated with nonspecific siRNA are considered as 1X sample) to determine the relative target levels (i.e., *n*-fold difference relative to the calibrator) (**Table 1**).

3.8. Trophozoite Crude Extract Preparation

1. Trophozoite crude extracts from each condition (i.e., wild-type trophozoites and trophozoites treated with either nonspecific siRNA or specific siRNA) are prepared using the following proportions: 5/10 of cell lysis buffer (125 µL/1 × 10^6 trophozoites), 1/10 of 10% SDS solution, and complete up to a final volume of 10/10 with 10 m*M* of Tris buffer.
2. Discard culture media supernatant from each tube, and gently rinse adherent trophozoites once with 2 mL of PBS previously incubated at 37 °C.

Table 1
Standard Procedures to Calculate Target mRNA Fold Change Based on Relative Standard Curve Method Using Real-Time PCR Data*

Step	Action
1	Collect real-time PCR data (i.e., CTs) into EXCEL data sheet.
2	Calculate average CT for each amplification triplicate including unknown experimental samples, target and endogenous standard curves.
3	Plot trendline for each standard curve (dilution on *x*-axis and average CT on *y*-axis).
4	Deduce trendline equations using EXCEL tools (i.e., $y = mx + b$).
5	Calculate target and endogenous amount for unknown samples using average CT and the following formula using appropriate slope and *y*-intercept from either target or endogenous trendline equation:[Target or endogenous amount] = $10^{([\text{average CT value}]-b)/m}$, where b is *y*-intercept and m is the slope of the standard curve trendline.
6	Divide target amount by endogenous amount to obtain a normalized target value.
7	Calculate the average target amount for each set of cDNA serial dilutions (i.e., three 10-fold dilutions of cDNA synthesis used as amplification template for each experimental condition).
8	Divide each of the normalized average target values by the calibrator normalized average target value (i.e., control trophozoites treated with nonspecific siRNA = 1 × level) to determine the relative target levels for each experimental condition.

*A reliable quantification is obtained when both standard curve trendlines (target and endogenous reference) have a similar slope ≥ 3.2 with a correlation coefficient ≥ 0.9 (step 4). The average CT from each experimental unknown sample should fit within both standard curve ranges (step 2).

3. Discard supernatant from rinse and immediately add 5 mL of fresh PBS to each culture tube. Detach adherent cells by striking the bottom of tubes three times against the workbench surface. Gently mix several times by inverting the tubes, and immediately collect an aliquot from each tube. Determine the cellular density for each aliquot by counting the number of cells with a Malassez chamber.
4. While counting the cellular density for each aliquot, centrifuge the culture tubes at 300g for 5 min at 4 °C. Discard the supernatant and keep the tubes on ice. Calculate the total number of cells per tube using the cellular density, and immediately add 125 µL of cell lysis buffer per 1×10^6 trophozoites (i.e., 5/10 volume; adjust proportionally to the number of trophozoites).
5. Using an automatic micropipette, measure the volume of the suspension (i.e., trophozoites + cell lysis buffer) in each culture tube and record values (cell

suspension). Transfer each cellular suspension to a microcentrifuge tube and add 25 µL of 10% SDS solution (i.e., 1/10 volume). Calculate the arithmetic sum of the cell suspension volume plus 10% SDS volume. To each tube containing the cell suspension plus SDS, add a suitable volume of 10 m*M* Tris buffer up to a final volume of 10/10 (i.e., 250 µL).

6. Boil the samples in a heater block at 105 °C for 5 min. Cool down the samples for 5 min on ice and spin down briefly to collect all the volume at the bottom of the tubes. Store each crude extract in single-use aliquots at –20 °C. To prepare samples for SDS-PAGE, mix 10 µL of crude extract (i.e., 4×10^4 trophozoites per lane) with 10 µL of 2X loading buffer and boil for 5 min at 105 °C. Spin down the tubes and cool the samples ~5 min at room temperature before loading on the gel.

3.9. SDS-PAGE

1. Clean glass plates with detergent and extensively rinse with distilled water. Assemble a glass-plate sandwich with 1.5-mm spacers and place the sandwich in a casting frame. Insert a comb into the glass-plate sandwich and make a mark on the glass with a nonpermanent marker, approximately 0.5–1 cm below the edge of the teeth.
2. Prepare 10% separating gel by mixing 6.7 mL of acrylamide/bisacrylamide solution, 6.7 mL of 4X separating buffer, 8.3 mL of water, 120 µL of ammonium persulfate solution, and 20 µL of TEMED. Immediately pour separating gel up to the mark on the glass, and overlay with isopropanol. Wait for polymerization of the gel (approx. 20–25 min) and rinse away isopropanol overlay with distilled water. Drain any excess water with a piece of filter paper, without touching the top of separating gel.
3. Prepare the stacking gel by mixing 0.8 mL of acrylamide/bisacrylamide solution, 1.25 mL of 4X stacking buffer, 3 mL of water, 50 µL of ammonium persulfate solution, and 6 µL of TEMED. Immediately pour stacking gel and insert the comb. Avoid air bubbles at the bottom of the comb. Stacking gel should polymerize within 10–15 min.
4. Carefully remove the comb, and place the gel sandwich into the electrophoresis unit. Wash the wells with running buffer using a 200-µL automatic micropipette, and fill the upper chamber with running buffer.
5. Check that no running buffer is leaking from the upper chamber. Load the samples and molecular marker into the wells using fine micropipette tips or a microsyringe. Add running buffer to the lower chamber until the lower electrode is completely submerged (~1 cm above the bottom of the gel sandwich).
6. Place the safety lid on the unit and plug the leads into the jacks of the power supply, with proper orientation (anode to anode in red, and cathode to cathode in black). A constant current of 20 mA per gel is recommended to run the gel (~1 h). Turn off the power supply once the tracking dye reaches the bottom of the gel, and immediately proceed to the blotting.

3.10. Western Blotting for Target Protein

1. Molecular marker and crude extract proteins resolved by SDS-page are transferred to a PVDF membrane. Cut a piece of PVDF and two pieces of 3MM paper the size of an electrophoresis sandwich (wear gloves when handling the membrane and gel to prevent contamination).
2. Soak PVDF membrane in methanol for 5 min and rinse in transfer buffer. Equilibrate the gel and soak fiber pads in transfer buffer for 5–10 min before the blotting.
3. Assemble the blot sandwich into the transfer cassette with dark side down, submerged in a tray filled with transfer buffer (enough to lay out transfer cassette). Sandwich elements are laid over the dark side of the cassette in the following order: a prewetted fiber pad, a piece of 3MM paper, the equilibrated gel, the prewetted PVDF membrane, a piece of 3MM paper, and a prewetted fiber pad. Use a glass tube to gently roll air bubbles out, and lock the cassette with the white latch.
4. Place a magnetic stir bar, the transfer module, and the cooling unit (previously filled with water and frozen at –20 °C) into the transfer tank. Fill the tank with transfer buffer (~800 mL) and place the cassette into the transfer module with the white latch up and the dark side facing the dark side of the module.
5. Place the tank over a magnetic stirrer, and close the unit with the protection lid. Plug the leads into the jacks of the power supply, with proper orientation (anode to anode in red, and cathode to cathode in black). The transfer can be accomplished at either 15 V overnight or 80 V for 2 h.
6. Once the transfer is complete, the cassette is removed from the transfer module and carefully disassembled to recover the PVDF membrane. The prestained molecular marker should be clearly visible on the membrane (*see* **Note 4**).
7. The PVDF membrane is incubated in 50 mL of blocking buffer for 1 h at room temperature on an orbital shaker. Discard the blocking buffer and briefly rinse the membrane in washing buffer.
8. Dilute the primary antibody in blocking buffer to a suitable concentration. Incubate the membrane in diluted primary antibody for 1 h at room temperature on an orbital shaker. The primary antibody is then removed and the membrane is washed 5 × 5 min with 50 mL of fresh changes of washing buffer at room temperature with shaking.
9. Dilute the secondary antibody in washing buffer to a suitable concentration (usually 1:20,000). Incubate the membrane in diluted secondary antibody for 30 min at room temperature on an orbital shaker. The secondary antibody is discarded and the membrane is washed as before.
10. During the final wash, equilibrate detection reagents at room temperature and mix 4 mL of solution A with 100 μL of solution B. Place the membrane with protein side up on a sheet of Saran Wrap and overlay with detection mix. Incubate for 5 min at room temperature.

11. Hold the membrane with forceps vertically for a few seconds to drain the excess detection mix and transfer to a fresh piece of Saran Wrap large enough to wrap up the membrane. Gently smooth air bubbles out and place the wrapped membrane, protein side up, in an X-ray film cassette.
12. Inside a dark room using red safe lights, place a sheet of autoradiography film on top of the wrapped membrane. Expose the film for 15 sec. Remove the film and develop immediately using an automatic developing device (alternatively, the film can be developed manually with developing and fixing solutions using standard procedures). Estimate the time of a second exposure if necessary on the basis of the appearance of the initial developed film.
13. The same membrane can be reused to assay other antibodies by removing primary and secondary antibodies. Remove the membrane from the Saran Wrap and incubate in stripping buffer for 30 min at 55 °C with occasional agitation. Wash the membrane twice for 10 min with 50 mL of washing buffer at room temperature on an orbital shaker. Incubate the membrane with blocking buffer as described above and repeat immunodetection.

4. Notes

1. RNA oligonucleotides are highly susceptible to degradation by exogenous RNases introduced during handling. Therefore, it is important that all handling steps be conducted under sterile, RNase-free conditions. Upon receipt, dried RNA oligonucleotides may be safely stored for up to 6 months at –20 °C. Once annealed, duplex siRNA is much more nuclease-resistant than single-stranded RNA.
2. We have observed that for some *E. histolytica* target genes, a longer duration of siRNA soaking up to 36 h improves the levels of gene silencing. A time-course siRNA soaking experiment could help to determine the optimal timing for gene silencing.
3. As a control for further amplification procedures (i.e., real-time PCR), an identical RT-PCR per condition can be set up with nuclease-free water instead of reverse transcriptase (non-retrotranscribed RNA).
4. Once the proteins are transferred to the PVDF membrane, it is important not to allow the membrane to dry during subsequent steps since this could interfere with immunodetection.

Acknowledgments

Thanks are due to Laurence Vayssié, who initiated the RNAi project in our laboratory. This work was supported in part by a grant from the European Union INCO-DEV program (ICA4-CT-2001-10073) and a postdoctoral fellowship from Fondation pour la Recherche Médicale (FRM-ACE20050703979).

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