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RNA Interference Using a Plasmid Construct Expressing Short-Hairpin RNA

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Summary

RNA interference (RNAi) is one of the most commonly used procedures for gene targeting in today's cutting edge technology and has great potential for use in clinical therapy. Using a plasmid construct that exogenously expresses short-hairpin RNAs (shRNAs) targeting a desired gene transcript not only helps to study the downstream effects of a gene product but also offers an alternative to viral vectors for gene therapy. Using a plasmid vector to knockdown a gene allows for long-term and permanent gene knockdown, without the need to generate knockout genotypes. Here, we detail the methodology for constructing a plasmid targeting the human telomerase reverse transcriptase (*hTERT*) gene through RNAi using the Ambion pSilencer system.

Keywords

Plasmid; RNAi; shRNA; human embryonic kidney cells; hTERT

1. Introduction

Generating knockout genotypic cell lines can be difficult and time consuming. Since the discovery of RNA interference (RNAi), knocking down a gene to prevent the translation of its transcript is an easier alternative to study the downstream effects of that gene as compared with the more traditional methods of gene targeting. RNAi can be performed in multiple ways. Here, we show the method of knocking down human telomerase reverse transcriptase (*hTERT*) gene using a plasmid construct that expresses a transcript that forms a short-hairpin RNA (shRNA).

One way to decrease the amount of non-specific knockdown effects of RNAi (1,2) is to construct a short 19-mer target of the mRNA from the desired gene. The use of a shorter target sequence reduces the chance of sequence homology between the target transcript and other gene transcripts as compared with the use of long double-stranded RNA. Multiple targets should be selected because the effects of RNAi are variable depending on target sequences, cell types, and mode of delivery into the cells. Another important factor is the structure of the target sequence mRNA, which affects accessibility of the RNA-induced silencing complex (RISC) or the binding of the complementary shRNA (3).

The gene-specific insert is constructed to transcribe a hairpin structure consisting of a 19 bp sequence from the target transcript, a spacer of 9 bp of random sequence for the hairpin loop, followed by the reverse complement of the original 19 bp sequence and a RNA polymerase II termination sequence consisting of a series of thymine nucleotides (4) (*see* Fig. 1). The plasmid construct should include either a H1 or a U6 promoter element at the 5′ end of the insert to allow RNA polymerase II recognition and binding, for the transcription of the insert. Intrinsically, RNA polymerase II transcribes mRNA and small nuclear RNAs. To reduce off-target effects, the 9 bp random hairpin sequence should contain no sequence homology to any

gene transcript, and these sequences can be obtained from several commercial sources (5) or by checking its homology to other transcripts at http://www.ncbi.nlm.nih.gov by using the blast search.

The use of plasmid constructs for stable RNAi is advantageous over retroviral constructs because there is no need for concern of random integration into the genome. However, there is a risk that the cells may tend to exclude the plasmid vector after long-term cell culture. The long-term knockdown of *hTERT* is a useful tool in analyzing the role of telomerase on differentiating or cancerous systems, because telomerase activity is up-regulated in most cancer cell types. As telomeres are also thought to act as a biological clock, inducing the repression of hTERT by knocking the gene down using plasmid RNAi can prove useful in studying the developmental and aging aspects of different cell types.

2. Materials

2.1. Cell Culture

1. HEK 293 cells (cat. no. CRL-1573, ATCC, Manassas, VA).
2. HEK medium: Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (HyClone, Ogden, UT).
3. Solution of trypsin (0.25%) and ethylenediamine tetraacetic acid (EDTA) (1 mM) (Invitrogen Corporation).

2.2. Plasmid Construct

1. pSilencer 3.1 H1-neo kit (Ambion, Austin, TX), containing plasmid with H1 promoter and neomycin/ampicillin antibiotic resistance.

2. Oligonucleotide sense sequence: *Bam*H1 restriction site/19-mer target sequence/9 bp loop/19-mer reverse complementary sequence/TTTTTT/*Hind*III restriction site.

4. Oligonucleotide antisense sequence.

5. Restriction endonucleases *Hind*III 10U/μl and *Bam*H1 10U/μl (Roche Diagnostics Corporation, Indianapolis, IN).

6. Restriction digestion buffer: 10× SURE cut buffer B (Roche Diagnostics Corporation) [100 mM Tris–HCl (pH 8.0), 50 mM $MgCl_2$, 1000 mM NaCl, 10 mM 2-mercaptoethanol].

7. T4 DNA ligase, 3 U/μl (Promega Corporation, Madison, WI).

8. 10× T4 DNA ligase buffer (Promega Corporation) [300 mM Tris–HCl (pH 7.8), 100 mM $MgCl_2$, 100 mM dithiothreitol (DTT), 10 mM ATP].

9. QIAquick DNA cleanup systems (Qiagen, Valencia, CA).

2.3. Bacterial Culture

1. Max efficiency DH5α competent *Escherichia coli* cells (Invitrogen Corporation).
2. Luria Broth medium, ampicillin 100μg/ml.
3. Luria Broth medium agar plates, ampicillin 100μg/ml.
4. Ligated plasmid with insert.
5. QIAprep Spin Miniprep Kit (Qiagen).

2.4. Transfection and Selection

1. Fugene 6 transfection reagent (Roche Diagnostics Corporation). Store at −4°C.
2. Purified plasmid clones.
3. DMEM. Pre-warm at 37 °C before transfection.
4. 100-mm tissue culture plates.
5. HEK selection medium: DMEM, 10% FBS, 300μg/ml neomycin G418 antibiotic (cat. no. A1720, Sigma, St. Louis, MO).

3. Methods

This protocol is specially designed for the construction of a plasmid vector using the pSilencer system from Ambion (*see* Note 1). There are, however, other compatible plasmids available for RNAi on the market such as fluorescence-producing or inducible systems (*see* **Notes** [2] and [3]). It should be noted that many commercially obtained plasmids may be sold as linearized plasmids, but it is always good practice to ensure that the plasmids are linearized and do not contain any foreign inserts. This can be done by digesting with the appropriate restriction enzymes and purifying the plasmid.

After selecting and picking clones, it is advisable to check for the presence of the insert by PCR amplification or DNA sequencing. DNA sequencing would also determine whether the insert contains any mutations because of cloning artifacts.

3.1. Annealing Oligonucleotides

According to the manufacturer's instructions,

1. Anneal equal volumes (2μl each) of the sense and antisense siRNA target oligonucleotides at a concentration of 1μg/μl each with 46μ l× DNA annealing solution provided with the pSilencer 3.1 H1-neo kit.
2. Incubate at 90 °C for 3 min to ensure that the oligonucleotides are not folding on themselves.
3. Incubate at 37 °C for 1 h.
4. Prepare a working stock of annealed oligonucleotides at a concentration of 8 ng/ml.
5. Store at −20 °C until ready for ligation.

3.2. Cloning into pSilencer Plasmid

1. Digest 1.5–2μg purified plasmid with 0.2μl BamH1 (10 U/μl), 0.2μl HindIII (10 U/μl), and 2.5μl 10× SURE cut buffer B and sterile deionized water to a final volume of 25μl. Add the enzymes last after mixing the solution gently to ensure that the enzymes do not denature because of improper salt conditions (*see* Note 4).

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[1]Plasmids with polymerase III promoters H1 or U6 can be used in the production of RNA transcripts because they have well-defined transcription start and termination sequences and do not produce poly-adenosine tails in their transcripts (6).

[2]It is sometimes useful to have a plasmid system that also confers green fluorescent protein (GFP) fluorescence to confirm the presence of the plasmid in the cell, especially for studies that involve observing phenotypic changes of targeted cells.

[3]Temperature sensitive or other inducible plasmid systems (such as tetracycline) may be used if the knockdown of a gene is toxic or fatal to the cells.

[4]When adjusting the conditions for multiple restriction enzyme digestion, ensure that the digestion buffer is compatible for the usage of all enzymes. Also, the total volume of the enzymes should not exceed 10% of the total volume of the reaction because of the fact that the glycerol content in the restriction enzyme stock may interfere with the digestion process.

2. Digest the mixture at 37 °C for 1 h and then inactivate the enzymes by incubating at 65 °C for 15 min.
3. Purify the digested plasmid with the QIAquick DNA Cleanup Systems kit. Follow the Nucleotide Removal Kit Protocol and elute with 20μl Elution buffer (EB) [10mm Tris-ce, pH 8.5] buffer. Store at −20 °C until ready for ligation.
4. To clone the annealed insert into the plasmid, add 4μl diluted insert, 1.33μl nuclease-free water, 2μl digested plasmid, 1μl 10× T4 DNA ligase buffer, 1.67μl T4 DNA ligase. Be sure to add the T4 DNA ligase last.
5. Ligate at 15 °C for 16 h.
6. Store at −20 °C until ready for transformation.

3.3. Transformation

1. Thaw DH5α competent *E. coli* cells on ice.
2. To 25μl aliquots of competent cells, add 4.5μl ligation reaction and mix by gently flicking the microfuge tube.
3. Incubate the mixture on ice for 30 min.
4. Place 37 °C water bath for 45 s for heat shocking.
5. Incubate on ice for 2 min, being careful not to mix or shake the mixture.
6. Add cells to 950μl pre-warmed Luria Broth medium with ampicillin.
7. Incubate in a 37 °C shaker for exactly 1 h. Secure the microfuge tube horizontally in the shaker to ensure that the cells receive adequate oxygen for maximal growth.
8. Plate the transformed cells on Luria Broth plates with ampicillin. It is useful to inoculate 100 and 200μl the reaction to obtain enough isolated clones. The remaining transformed cells may be kept at 4 °C for further inoculation on Luria Broth agar plates if necessary.
9. Incubate the plates inverted at 37 °C overnight.

3.4. Clone Amplification

1. Pick isolated colonies and inoculate each clone into 3 ml aliquots of Luria Broth with ampicillin.
2. Incubate in a 37 °C shaker for 16 h. A glycerol stock of the individual bacterial clones can be made and stored for future use (*see* **Notes** [5] and [6]).
3. Pellet the cells by centrifugation. Aspirate the supernatant.
4. Wash 1× with 700μl phosphate-buffered saline (PBS) and pellet by centrifugation. Aspirate the supernatant.
5. Store at −20 °C until ready for miniprep.
6. Follow the manufacturer's instructions using the QIAspin Miniprep Kit and elute with 50μl sterile deionized water. It is important to use water for elution instead of the

[5]Store transformed bacteria with desired clones in 15% glycerol/cell suspension in Luria Broth. Snap freeze in liquid nitrogen and store at −80 °C.

[6]When growing bacteria in Luria Broth suspension, it is not advisable to inoculate directly from a frozen stock because of purity and yield concerns. Instead, streak on Luria Broth agar and incubate for 24 h at 37 °C, then inoculate from an isolated clone.

provided buffer from the kit because it might interfere with PCR or DNA sequencing procedures.

7. Each clone can be sent for DNA sequencing or PCR to confirm the presence of the insert.

3.5. HEK Cell Culture and Transfection

1. HEK 293 cells obtained from ATCC are cultured with HEK medium at 37 °C and 5% CO_2. They are maintained in 100-mm tissue culture dishes and passaged with trypsin/EDTA at 80% confluency to maintain log phase of cell growth (especially for contact-inhibited cell lines), because transfection efficacy depends highly on the phase of cell growth. HEK cells are usually split 1:10–1:12 with each passage.
2. HEK cell cultures should be no more than 60% confluency on the day of transfection.
3. If antibiotics are used for cell-culture maintenance, the culture should be washed 1× with pre-warmed HEK medium without antibiotics and replaced with fresh medium before transfection (*see* Note 7). Antibiotics should not be added to the culture medium during transfection with Fugene 6 transfection reagent because the influx of antibiotics with the transfection reagent into the cells is highly toxic.
4. 10–11μg plasmid is required per 100-mm dish. Fugene 6 transfection reagent is added to pre-warmed DMEM (37 °C, without serum) at a ratio of 1:5 (microgram : microliter Fugene). Add the transfection reagent carefully into the middle of the microfuge tube to avoid the reagent from contacting and sticking to the walls of the tube. The appropriate amount of plasmid is carefully pipeted into the middle of the tube and should appear as a clear liquid in a contained suspension with the Fugene 6 transfection reagent. Incubate the mixture unshaken for at least 15 min at room temperature.
5. Vortex the transfection mixture briefly to obtain an even suspension and add the appropriate amount around the well in a drop-wise manner. Incubate at 37 °C for 24–48 h.
6. Replace the HEK medium with HEK selection medium. Maintain and refresh the HEK selection medium every 3 days. HEK G418-resistant cells will persist and proliferate (*see* Note 8). Split the cell culture as needed.

References

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[7]Washing HEK cells on the culture plate should only be done with pre-warmed HEK medium. Using PBS will only cause the cells to lose adherence and lift off the plate.
[8]Selection of transfected HEK cells on HEK selection media should continue for at least 2 weeks before the cells may be harvested to ensure thorough selection of cells.

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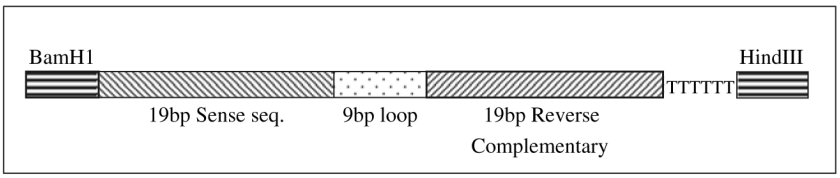

Fig. 1.

Construct of a typical short-hairpin RNA (shRNA) vector insert, 5′–3′. Different restriction sequences are placed on the 5′ and 3′ ends. A 19 bp sequence for the target mRNA (sense sequence), 9 bp stem loop, and a 19 bp reverse complementary of the target sequence. When transcribed, the insert will form a secondary hairpin structure to cause the knockdown of the target gene.