Chapter 16

Design and Construction of Shrimp Antiviral DNA Vaccines Expressing Long and Short Hairpins for Protection by RNA Interference

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1 Introduction

DNA vaccines are essentially recombinant plasmid constructs capable of expressing pathogen-derived antigenic proteins that prime the host against future infection when administered intramuscularly or subcutaneously [1–3]. DNA vaccines present the aquaculture industry with an effective and economically viable method of checking the threat of various pathogens that drastically affect productivity. They are considered safer compared to live, attenuated, and whole inactivated vaccines and are more stable than protein/glycoprotein subunit vaccines. However, it is for the viral and parasitic diseases that they are particularly attractive options [4]. The DNA vaccine against infectious hematopoietic necrosis virus (IHNV) that affects salmonid fishes is most effective and the only one licensed for use in aquaculture since 2005 [5]. In invertebrates like shrimps, however, the specific immune response system is rudimentary [6] and although there are some reports on application of subunit vaccines [7, 8] and DNA vaccines expressing viral proteins [9], they are of limited efficacy. The discovery of RNA interference (RNAi) pathway in shrimps provided a promising new approach to vaccination, and in current times, the definition of DNA vaccines can be extended to include plasmid constructs that express short or long double stranded RNA (dsRNA) in the host and inhibit pathogen proliferation through RNA interference mechanism.

In a dramatic discovery in 1998 it was found that dsRNA introduced into a eukaryotic cell results in silencing of the corresponding RNA transcript [10], a phenomenon that has been named

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"posttranscriptional gene silencing" (PTGS) or "RNA interference" (RNAi). The presence of dsRNA in the cytoplasm (whether it is transfected or synthesized within the cell) triggers the multidomain ribonuclease III enzyme Dicer [11]. This cleaves dsRNA into small interfering RNAs (siRNAs), which are 21–23 nucleotide fragments with characteristic 2-nucleotide 3′ overhangs. These siRNAs are recognized by the RNA-Induced Silencing Complex (RISC; [12]), a multienzyme unit that brings about separation of the two siRNA strands. The antisense siRNA strand remains bound to RISC, while the sense strand is released. In some organisms that have functional RNA-dependent RNA polymerase (RdRp) enzyme, the sense strand may be again converted into dsRNA [13]. The antisense strand guides RISC to bind the homologous (target) mRNA, and another RNase III Argonaute that is part of the complex cleaves it, silencing its expression [14]. The efficiency of siRNA depends on perfect complementarity of the seed sequence (positions 2–6) with the target mRNA. It has been reported that imperfect base pairing that creates a bulge in miRNA/siRNA marks the transcript for translational repression, while a bulge in the mRNA does not prevent cleavage [15]. It has recently been suggested that translational inhibition involves mRNA decapping that ultimately leads to its degradation in P bodies [16, 17].

PTGS, which perhaps evolved as a defense mechanism against RNA viruses [18], has been shown to exist in several plants and animals [19, 20]. The discovery of small genes coding for microRNA (miRNA; short hairpin shaped RNA molecules) that target specific mRNA transcripts also shows that the phenomenon is used to regulate gene expression [21, 22]. RNA interference caught the imagination of researchers all over the world as it opened a completely new box of molecular tools and applications. Aquaculture sector has not remained untouched by this excitement and several attempts are being made to check shrimp viral pathogens [23, 24] by triggering RNAi through exogenous antiviral long dsRNA and siRNA [25–29], or plasmid DNA constructs capable of expressing these molecules in vivo [30, 31]. The presence of Dicer gene has been reported in a number of decapods including *Penaeus monodon* [32, 33], *Litopenaeus vannamei* [34, 35], *Fenneropenaeus chinensis* [36] *Marsupenaeus japonicas* [37, 38], confirming the presence of a functional RNAi pathway.

The fate of plasmid DNA administered to fish by intramuscular/intraperitoneal injection, gene gun or orally has been explored by various researchers in salmon [39–42], Atlantic cod [43], rainbow trout [44–46], marine tiger shrimp *Penaeus monodon* [9, 30]. In fish, despite degradation at the site of administration and in blood plasma, cellular uptake and wide tissue distribution of the plasmid DNA has been observed by PCR, fluorescent in situ hybridization (FISH), isotope and fluorescent labeling [47]. Nevertheless, no histopathological damage was detected in rain-

bow trout up to 2 years after DNA vaccination against IHNV [48]. Detailed reports are not available from shrimp, but plasmid DNA vaccine injected intramuscularly between second and third abdominal segments could be amplified from several tissues after 30 days [30] and injected plasmid was shown to persist up to 2 months [9]. Das et al. [31] could observe no difference in growth rates of *P. monodon* treated with DNA vaccine expressing antiviral lhRNA.

There are several ways by which siRNA could be generated to silence a target gene using RNA interference technology. These include chemical methods where a 21 nt dsRNA is obtained by chemical synthesis or in vitro transcription and biological methods where a long or short hairpin RNA is generated from a plasmid vector in vivo that eventually is cleaved into 21 nt siRNA by dicer. Although siRNA has been the prime choice for gene silencing among several researchers across the globe, long hairpin RNA has the advantage that Dicer can act on it to generate a number of different siRNAs ensuring a robust RNAi effect [49–51]. In addition, it considerably reduces the chances of viral escape by point mutation [52]. The use of long dsRNA is avoided in mammals where it is known to induce a nonspecific interferon response leading to inhibition of protein translation [53], but it is possible in invertebrates and plants [54, 55], where interferons have not been detected [56]. The convenience of producing lhRNA/shRNA in vivo using host machinery cuts down the cost of production compared to chemical synthesis and in vitro transcription methods. Therefore, plasmid DNA constructs designed to express lhRNA/shRNA in vivo can be used as DNA vaccines.

1.1 Target Gene Selection

The choice of target viral gene is critical and its silencing should inhibit viral spread without causing any deleterious effect to the host organism [57]. If silencing of a single viral gene does not provide sufficient protection against infection, multiple viral genes can be targeted in a combinatorial RNAi therapy [58]. This approach can also prevent viral escape through point mutation [59] and it is noteworthy that RNA viruses accumulate point mutations up to 10^7-fold more rapidly than DNA viruses [60]. However, the dose of RNAi molecules should be regulated so as not to saturate/overload the endogenous RNAi pathway with too many siRNAs [57]. In some cases it is also possible to target host factors that are essential for viral propagation, provided host cell viability is not affected [24].

1.2 Identification of Target Sequence and Design

Characteristics of the specific sequence to be targeted within a selected gene have been suggested [61]. Broad guidelines include the following: (1) confirmation of sequence uniqueness by BLAST homology tool to prevent off-target effects; (2) the sequence must be conserved among different strains reported for a particular virus; (3) the selected sequence must have an optimal thermodynamic profile for incorporation into the RISC as a guide strand

The most effective siRNAs have a relatively low Tm and duplex stability (less stable, more A/U rich) toward the 5′-end of the guide strand and a relatively high Tm (more stable, more G/C rich) toward the 5′-end of passenger strand [61]; (4) the sequence should lie in the coding region, 50–75 bp away from the 5′ and 3′ ends [62, 63]; (5) mutation prone regions should be identified if possible and avoided. Many free online web tools are available for designing optimal RNAi molecules (e.g., E-RNAi: http://e-rnai.dkfz.de, Arziman et al. [64]). The software predicts siRNA efficiency using an algorithm described by Reynolds et al. [65].

1.3 Vector Selection

The selection of plasmid vector depends on the choice of promoter to be used for the expression of sh/lh RNA. Several vectors designed specifically for RNAi studies are commercially available (*see* Subheading 4). Generally, pol III promoters such as U6 and H1 are used for expressing shRNA constructs as they are compact, support high levels of transcription and initiate transcription at a defined starting point. Transcription terminates at a stretch of thymidines and 3′ terminus of the hairpin resembles a pre-miRNA. They express constitutively in cell culture, but expression from H1 is associated with dividing cells in vivo. There are no tissue specific pol III promoters that might be desirable for some transgenics. In such cases, use of pol II tissue specific promoters is a better option. Some constitutive pol II promoters that are active in shrimp include cytomegalovirus immediate early promoter (CMVp), β-actin and SV40 early promoters [66]. CMVp cannot be used for shRNA vectors as they append 5′ vector sequences and 3′ poly A sequences, which inhibit shRNA function, but are ideal for lhRNA constructs. For shRNA expression, pol III promoters like U6 and H1 are generally used. Bidirectional promoter constructs with U6 and H1 promoters are also developed for expressing two different shRNA molecules targeting two different genes or two gene segments of a particular gene of interest in order to increase the efficiency of targeted gene silencing (*see* Subheading 4).

Since a vaccine is intended for commercial use selection markers present an important consideration. Antibiotic markers are discouraged by regulatory authorities and it is wise to opt for non-antibiotic selection markers [67, 68]. Centre for Biologics Evaluation and Research (CBER), USA permits the use of kanamycin selection because resistance to this antibiotic is widespread [69].

1.4 Cloning Strategy

In case of lhRNA, the selected portion of the target viral gene is PCR amplified using specific linker primers containing appropriate restriction enzyme (RE) recognition sites for directional cloning in the vector of choice. The same fragment is cloned in reverse orientation leaving a spacer region of 5–7 bp for formation of hairpin loop (Fig. 1). Short-hairpin RNAs, on the other hand, are short stem-looped RNAs of size 19–23 bp. In this case, the complementary

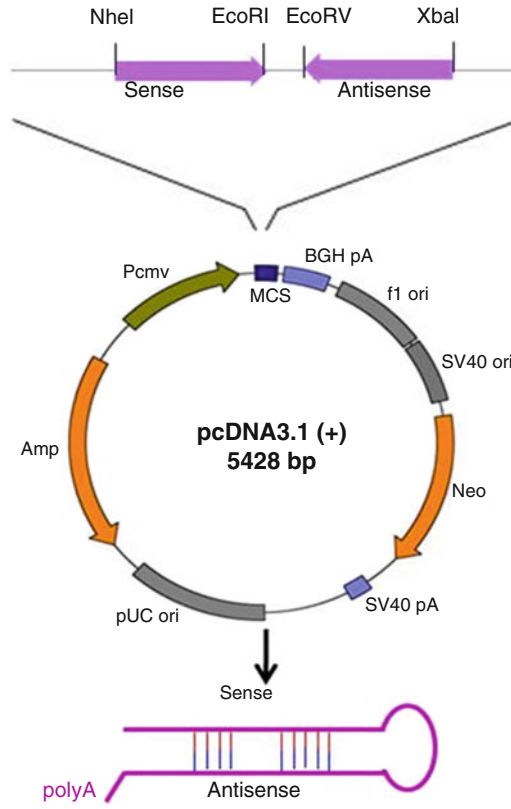

Fig. 1 Schematic diagram of lhRNA expression plasmid

strands can be synthesized and allowed to anneal at room temperature. The resulting cassette is designed to have appropriate overhangs to allow directional cloning in the selected vector (Fig. 2).

1.5 Assessment of Silencing Efficiency

Silencing efficacy of sh/lhRNA constructs can be tested in vitro before commencing the in vivo pathogen challenge studies. Here, the host cells are co-transfected with a plasmid that constitutively expresses the target gene and the sh/lhRNA construct. The silencing efficiency of the RNAi constructs can be estimated at transcript level by real-time PCR of the target gene and at protein level by ELISA. It is best to carry out these studies in shrimp primary cell cultures or cell lines. Although primary cultures could be developed from different tissues of shrimp [70], cell lines are yet to be developed. Other invertebrate cell lines like those derived from insects, *C. elegans*, etc. (Sf9, Sf21, and Drosophila S2) can also be used. In vertebrates, lhRNA is known to induce nonspecific interferon response leading to inhibition of protein translation in general [54, 55] and this phenomenon may account for some silencing effect if vertebrate cell lines are used.

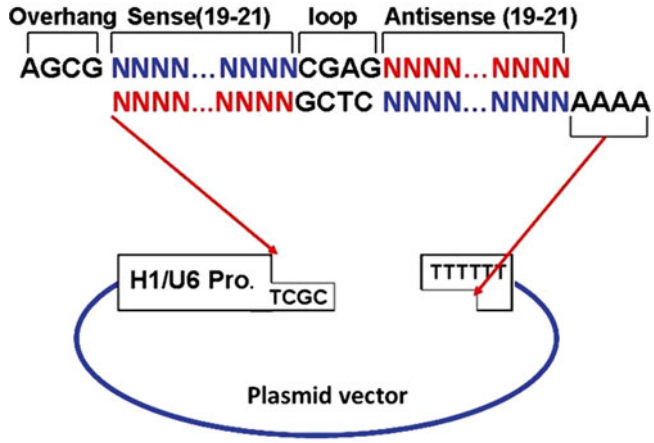

Fig. 2 Schematic diagram of shRNA expression plasmid

1.6 Challenge Studies for Estimating Protection from Pathogen

There are a number of ways in which DNA vaccine can be introduced into shrimps. Most RNAi-based therapeutic trials have been conducted on juveniles by intramuscular injection of plasmid into the abdominal segments [9, 30, 31, 71]. Dip treatment and oral administration [29, 72] are also possible, but plasmid DNA degradation and poor bioavailability from the gastro-intestinal tract are factors to be considered. The vaccinated and control animals are challenged with an appropriate titer of the pathogen and parameters such as survival, disease symptoms, histopathology, and viral load are recorded. The survival percentage is reported as the protection efficiency of the vaccine.

2 Materials

2.1 Target Sequence Selection

1. Bioinformatic tools for determining coordinates of target sequence within the target gene (Gene Runner v3.05, DNASTAR v12.2, etc.).
2. Online software for multiple sequence alignment (e.g., EBI Clustal W, MEGA v6.0).
3. Analysis of target gene using sh/lhRNA design software (E-RNAi, BLOCK-iT™ RNAi Designer, siRNA Wizard v3.1, SVM RNAi 3.6, siDESIGN Center, etc.).
4. National Center for Biotechnology Information (NCBI) BlastN online server.
5. Bioinformatic tools for DNA repeat analysis (e.g., RepeatFinder, Tandem Repeat Finder, Palindrome, Spectral Repeat Finder (SRF), RepeatMasker).

6. Online server tools for restriction site identification (e.g., NEBcutter, Webcutter 2.0, Watcut, Gene Runner).
7. Online server tools for RNA secondary structure prediction (e.g., RNAfold, Sfold, RNA123, RNAshapes).

2.2 Vector Selection

1. Lh-RNA expression: pcDNA3.1 series of vectors (Invitrogen, USA).
2. Sh-RNA expression: pSuper (Oligoengine, USA), BLOCK-iT™ shRNA Entry Vectors (Invitrogen, USA), pSilencer 2.1-U6 vector (Invitrogen, USA), pSIREN-U6 vector (Clontech, USA).
3. Target gene expression: pcDNA4/His-Max series of vectors (Invitrogen, USA).

2.3 Construction of sh/lh-RNA Expression Plasmid

1. PCR components: Template DNA (50–100 ng/μL), 10 pmol of each specific primer, 200 μM of each dNTPs, 0.75 units of *Taq* DNA polymerase and 1× *Taq* buffer containing 1.5 mM $MgCl_2$.
2. Selected restriction enzymes and 10× buffers.
3. Hybridization buffer for annealing complementary oligonucleotide strands: 25 mM Tris–HCl (pH 8.0) and 10 mM $MgCl_2$.
4. T4 DNA ligase and 10× buffer.
5. Horizontal agarose gel electrophoresis apparatus and power pack.
6. Agarose gel: For 100 mL of 1.0 % agarose gel, use 1.0 g of agarose (electrophoresis grade) with 100 mL of 0.5× TAE. Prepare 1 L of 10× TAE stock solution in ultrapure water with 48.4 g of Tris base, 3.72 g disodium EDTA, and adjust to pH 8.5 with glacial acetic acid.
7. Agarose, 6× gel loading dye, and nucleic acid stains.
8. Gel documentation system.
9. Genomic DNA, total RNA isolation and cDNA synthesis kits.
10. Gel extraction and plasmid miniprep kits.
11. *E. coli* DH5α competent cells (available commercially).
12. Water bath/incubator.
13. Luria Bertani medium: To 150 mL of double distilled water add 2 g Tryptone, 2 g NaCl, 1 g Yeast extract, 3 g Agar. Adjust the volume to 200 mL with double distilled water and sterilize by autoclaving at 121 °C and 15 psi for 15–20 min.
14. Bacteriological incubator for growing *E. coli* on plates.
15. Shaking incubator for growth of broth cultures of *E. coli*.
16. Sterile inoculation loops.

2.4 In Vitro Validation

1. Sf9 insect cell line or any other convenient invertebrate cell line.
2. Appropriate growth medium components for selected cell line.
3. PBS containing 1 % v/v penicillin–streptomycin.
4. Inverted microscope.
5. CO_2 incubator for growing cells.
6. Hemocytometer for cell counting before passaging.
7. Effectene® Transfection Reagent (Thermo Scientific, USA) or equivalent.
8. EndoFree® plasmid purification kit (Qiagen, NL) to isolate endotoxin free plasmid DNA for transfection.

2.5 Evaluation of sh/IhRNA Constructs In Vivo

1. Experimental shrimp (e.g., *Penaeus monodon*) of 10–12 g body weight.
2. 1000 L fiberglass tanks with seawater and aeration.
3. Kit for estimating dissolved oxygen, pH paper, thermometer, and salinometer for maintaining water quality parameters.
4. Artificial pellet feed.
5. Viral detection kits to select only healthy animals for experiments.
6. Titered viral inoculum to determine dose that results in complete mortality within 10 days.
7. Lh-RNA/sh-RNA expression construct.
8. 1 mL syringe with 20 G needle.

3 Methods

3.1 Target Sequence Selection

1. Retrieve the sequence information for the viral target gene in FASTA format from online databases like NCBI GenBank.
2. Perform multiple sequence alignment of the target gene obtained from different viral strains/isolates in Clustal W to identify the conserved region for silencing (*see* **Note 1**).
3. Remove 50–75 bp of the sequence from 5′ and 3′ ends of the target gene and perform BLASTn analysis to ensure that they do not share any significant homology with any known genes of the host organism (shrimp).
4. Analyze the sequence with online server tools to identify repeat sequences if any. These sequences can be removed if they are located towards the ends or else another target gene may be selected.
5. Free online software E-RNAi can be used for dsRNA molecule designing (*see* **Note 2**). On the E-RNAi web page select the RNAi type as 'long dsRNA' from dropbox.

6. Enable or disable the off-target evaluation option depending on whether whole genome and/or EST information is available for the experimental animal or not. No shrimp whole genome sequences are available at the time of writing this article (*see* **Note 3**). Copy and paste the selected region of the target gene in the box provided and click submit. On the '*De novo* design: settings' page, select the number of output sequences you want to be displayed (e.g., 5).
7. The output consists of the (a) target sequence options meeting the design criteria, (b) a set of primers for each output sequence, (c) a report on the siRNA efficiency (expressed as percentage of efficient siRNAs based on predetermined criteria), and (d) specificity (calculated as the number of matching siRNAs over the number of all siRNAs in the long dsRNA of interest).
8. Select the best primer set that amplifies a conserved region (*tar*) of the gene. This region is expected to produce the largest number of efficient siRNAs and recognize all known strains/isolates of the virus.
9. Perform restriction analysis of *tar* using Gene Runner software and select restriction sites that are absent in *tar* and present in the *mcs* of selected vector for use as linkers in the primers to facilitate directional cloning.
10. *Tar* is to be cloned in forward and reverse orientations in the *mcs* of the selected vector leaving a spacer region of ~5–10 bases. Restriction enzyme (RE) sites are to be selected keeping this in mind (e.g., in pcDNA 3.1(+) using *Hind*III and *Eco*RI for forward fragment and *Eco*RV and *Xho*I for reverse will leave a spacer region of 10 bp in between).
11. To confirm whether the cassette will form a hairpin after transcription, join the forward sequence of *tar*, spacer bases and reverse sequence, and analyze it in silico for the formation of hairpin structure using RNAfold software. The reverse orientation of *tar* can be obtained in Gene Runner v 3.05.
12. Add appropriate linker sequences to the selected primer set to clone *tar* in forward and reverse orientations. This will yield two sets of primers that can be synthesized chemically. In the above example for cloning the forward fragment F and R primers will have *Hin*dIII and *Eco*RI recognition sequences attached as linkers while for cloning in reverse orientation the same F and R primers will have *Xho*I and *Eco*RV.

3.2 Construction of Ih-RNA Expression Plasmid

1. If the target gene belongs to a DNA virus, *tar* can be amplified from genomic DNA isolated from tissues of an infected animal using a Genomic DNA isolation kit.
2. If the target gene belongs to a RNA virus, *tar* can be amplified from cDNA prepared from an infected tissue. For this total RNA can be isolated and cDNA prepared using kits.

3. PCR amplify *tar* in both orientations. Perform PCR in 25 μL reaction volume containing 50 ng template DNA, 10 pmol of each specific primer, 200 μM of each dNTPs, 0.75 units of *Taq* DNA polymerase and 1× *Taq* buffer containing 1.5 mM $MgCl_2$.
4. Load the PCR product in 2 % agarose gel and resolve along with a 100 bp DNA ladder.
5. Excise the desired band from the gel and purify *tar* using gel extraction kit following manufacturer's instructions.
6. RE digest 1 μg each of pcDNA3.1(+) vector and forward *tar*.
7. Resolve the digested vector and *tar* on 1 % and 2 % agarose gels, respectively. Excise the required bands and purify using a gel extraction kit (*see* **Note 4**).
8. Forward *tar* is ligated with the vector. Briefly, a 20 μL ligation reaction contains vector and insert mixed in 1:3 molar ratio, 2 μL of 10× reaction buffer and 1 μL of T4 DNA ligase. The ligation reaction is incubated at 16 °C overnight.
9. For transformation, add 2 μL of ligation mixture to the 100 μL of *E. coli* DH5α competent cells and incubate on ice for 20 min followed by heat shock at 43.5 °C for 50 s. Immediately replace on ice and add 1 mL LB broth. Recover the cells by shaking at 37 °C for 1 h. Plate the cells on LB-Amp-Agar plates aseptically and incubate at 37 °C overnight in an incubator.
10. Prepare master plate of the well isolated colonies. For this pick the colonies using a sterile inoculation loop and streak on a fresh LB-Amp-Agar plate and incubate overnight at 37 °C in an incubator. This will provide enough culture for screening.
11. Select 9–10 colonies and pick a minute quantity of culture with a sterile pipette tip and suspend in 10 μL of TE buffer (pH 8.0). Perform colony PCR using 1 μL suspended cells as template according to standard protocol. Either insert or vector specific primers may be used for amplification.
12. Load the PCR products on 2 % agarose gel and resolve along with 100 bp DNA ladder. Recombinant clones should result in the amplification of insert of the appropriate size.
13. Select 4–5 colonies of colony PCR positive clones and inoculate 2 mL of LB-Amp broth followed by incubation at 37 °C overnight in a shaking incubator.
14. Isolate plasmid DNA from the culture using a plasmid miniprep kit following manufacturer's instructions and determine the concentration by spectrophotometry (Nanodrop, biophotometer).
15. Sequencing primers provided in the cloning vector can be used for confirming the presence of the insert.
16. The reverse orientation of *tar* can be cloned into the above construct by following **steps 6–15**. This completes the synthesis of lhRNA silencing construct.

3.3 Construction of sh-RNA Expression Plasmid

1. Perform **steps 1–6** as described in Subheading 3.1, this time selecting 'siRNA' option on the E-RNAi webpage.
2. The output consists of the possible target siRNA sequences (19 bp) meeting the design criteria and an efficiency score based on predetermined criteria. The most efficient siRNA lying in the conserved region may be selected.
3. Join the forward siRNA sequence, 5–10 bp spacer region and siRNA sequence in reverse orientation in Gene Runner v 3.05. Copy the sense and antisense strands separately and paste in Notepad. Both sequences will get pasted in 5′–3′ direction. Add overhang sequences to anneal with cohesive ends created in the vector by selected REs (e.g., GTAC for *Kpn*I and AGCT for *Sac*I to the 3′ ends, because these enzymes generate 3′ overhangs). These final sequences can be synthesized chemically.
4. Generate a double-stranded cassette by annealing the two sense and antisense oligonucleotides. Mix approximately 40 μM of each oligomer in the buffer containing 25 mM Tris–HCl (pH 8.0) and 10 mM $MgCl_2$. Denature the primers by boiling for 5 min in a water bath and slowly cool down to room temperature to allow the formation of the cassette.
5. Digest 1 μg of pSUPER vector with selected REs (e.g., *Sac*I and *Kpn*I) and heat inactivate the enzymes by following manufacturer's instructions.
6. Prepare the construct by following **steps 7–15** of Subheading 3.2. This completes the synthesis of shRNA silencing construct.

3.4 Expression Construct of Target Gene

1. Design primers to amplify full length target gene for in-frame cloning in any selected pcDNA4 HisMax series of vectors. Add appropriate linkers to the primers for directional cloning. PCR amplify the target gene proceed as described in **steps 4–15** in Subheading 3.2. This will synthesize the target gene expression construct.

3.5 In Vitro Estimation of Silencing Efficiency

1. The day before transfection, freshly passage the cells with recommended split ratio, so as to get the desired confluency (60–80 %) after 24 h, which is ideal for optimal transfection. The confluency can be confirmed under a microscope and the cell count can be obtained by using a hemocytometer.
2. Isolate the lh/shRNA silencing constructs and target gene expression plasmid using EndoFree® plasmid purification kit (Qiagen, NL) in order to avoid cytotoxicity due to endotoxins that co-purify with plasmid DNA.
3. Transfect the cultured cells with lh/shRNA silencing construct along with the target gene expression construct in equimolar ratio following manufacturer's instructions and culture the cells for 24–48 h. As a positive control, transfect cells with

target gene expression construct along with the empty vector. For negative control, transfect cells with only the empty vector.

4. Harvest the cells post transfection and wash twice with PBS containing 1 % v/v penicillin–streptomycin.
5. Isolate total RNA from the transfected cells and prepare cDNA using oligo-dT primers.
6. Target gene silencing can be determined by quantifying the transcript by real time PCR using primers designed in a region other than *tar*.
7. The silencing effect may also be determined at protein level by western blotting using anti-His tag antibodies.
8. The sh/lh construct that results in higher silencing efficiency can be tested in vivo (*see* **Note 5**).

3.6 Evaluation of sh/lhRNA Constructs In Vivo

1. Maintain experimental shrimp in 1000 L fiberglass tanks with aeration at 27–30 °C with appropriate salinity and artificial pellet feed thrice a day.
2. Monitor the water quality parameters such as temperature, pH, salinity, and dissolved oxygen at weekly intervals.
3. Prior to use, screen the experimental animals for the presence of pathogens under study using viral detection kits to ensure that only healthy individuals are used for the experiment.
4. Inject the treatment group intramuscularly with lh/sh-RNA expression plasmid at the rate of 1 μg/g body weight in a 100 μL volume using a disposable 1 mL syringe.
5. Inject negative and positive control groups similarly with empty vector.
6. Challenge the treatment and positive control groups with viral inoculum that results in 100 % mortality in about 10 days. Negative control group is injected with PBS buffer.
7. Monitor the shrimp daily and document the mortality pattern in each group until 90 % of the animals in a particular batch die. Freeze the dead shrimp in −80 °C for further use.
8. Ascertain the cause of death in the experimental animals by histopathology or molecular diagnostic tools like PCR or ELISA (to confirm death is due to infection and not other causes).
9. The viral copy number may be estimated in the vaccinated and control animals by real-time RT-PCR to calculate the silencing efficiency.
10. The vaccine efficiency is reported in terms of percent survival of vaccinated animals over controls and percent reduction in viral copy number.

4 Notes

1. In order to identify mutation prone regions within the viral gene to be silenced all the sequences should be subjected to multiple sequence alignment. These regions should be excluded while identifying the target sequence.
2. Almost all commercial suppliers of siRNA consumables provide online design service for free.
3. The E-RNAi software also offers the off-target evaluation with genomic/transcript database of popular model organisms. Since such information is very meager for shrimp the best alternative available is to go for BLAST analysis.
4. Restriction enzyme digestion is not 100 % efficient and often results in contamination with uncut DNA. This is particularly important in case of plasmid DNA as uncut plasmid runs close to the cut plasmid if the plasmid size is more than 5 kb. Therefore, it is recommended to completely resolve the RE digested plasmid DNA on 1 % agarose to avoid cross contamination with uncut plasmid.
5. In spite of all precautions, some sequences may lead to unexpected toxicity in vivo and so a pragmatic approach would be to screen 4–5 sequences for each gene before choosing the most effective therapeutic construct for in vivo trials.

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