IV. General Considerations

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.

Figure 3 shows an overview of the procedure described in this User Manual, which provides protocols for siRNA oligonucleotide sequence design, annealing of siRNA oligonucleotides, ligation of annealed oligonucleotides into RNAi-Ready pSIREN, and transformation and transfection of pSIREN constructs.

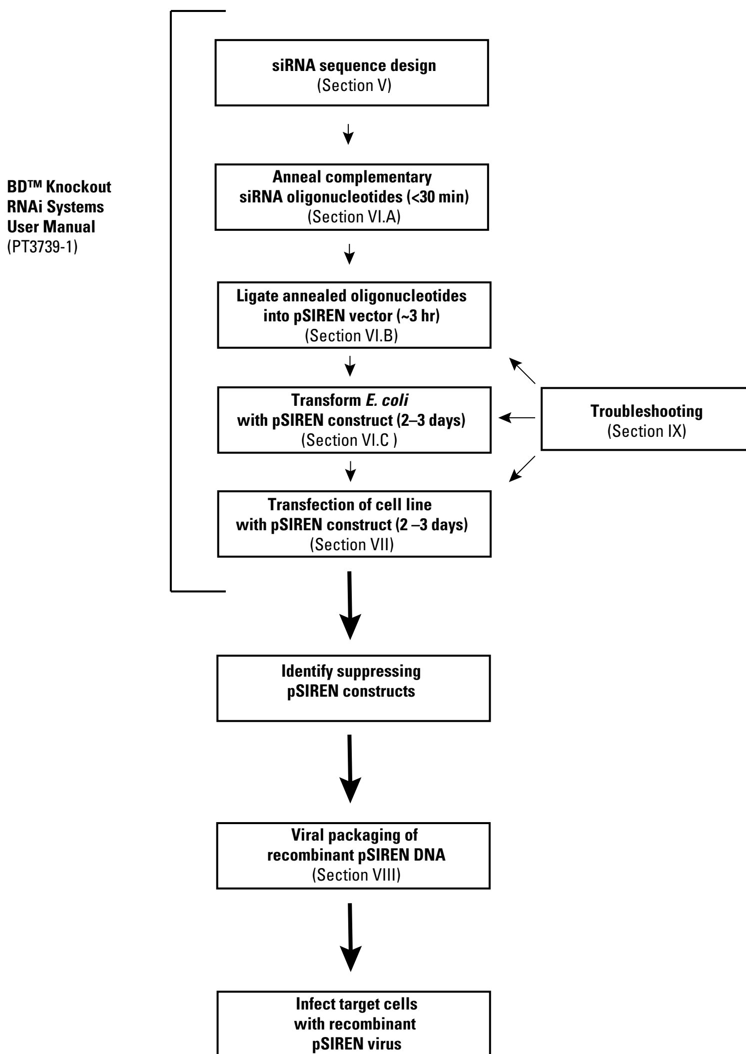

Figure 3. Overview of the BD™ Knockout RNAi Systems procedure.

IV. General Considerations *continued*

Protocols for recombinant pSIREN viral packaging, production, and infection are described in the secondary User Manual(s) that accompany your BD Knockout RNAi System (see Section VIII). We strongly recommend that you thoroughly read *all* User Manuals before beginning the procedure.

siRNA Oligonucleotide Design (Section V)

- The success of your experiment depends on choosing the proper target sequence within your gene of interest and the proper design of the siRNA oligonucleotides. In addition, we highly recommend that you test more than one siRNA sequence for a gene of interest.
- PAGE purification of your designed oligonucleotides ensures that a higher percentage of the oligonucleotides will be full-length and increases the chance of cloning a complete and functional insert. When using PAGE-purified oligonucleotides, we typically achieve 80–90% of clones with the right insert.
- When testing your pSIREN construct for functionality, you will need a gene-specific assay to test for the suppression of Gene X. Examples of gene-specific assays that can be used include:
    - Western blot with an antibody to Protein X
    - RT-PCR using Gene X primers. Be sure you can discriminate PCR products generated from genomic DNA from true RT-PCR products.
    - Northern blot with Gene X probe
    - Functional assay for Protein X

Transfection of Recombinant pSIREN Vectors (Section VII)

- The transfection protocol included in this User Manual is intended for the screening of functional siRNA constructs and gene silencing experiments using transfection. For performing gene silencing experiments using viral infection, please refer to the secondary User Manuals included with your BD Knockout RNAi System. The secondary User Manuals describe the protocols for viral packaging and infection using pSIREN constructs.
- If a transfection method is already established for your cell line model, proceed with those conditions. It is important to keep optimized parameters constant to obtain reproducible results.
- To ensure the purity of the DNA, isolate all plasmids for transfection using a NucleoBond Plasmid Maxi EF Kit (Cat. No. 635953) or by banding on a CsCl gradient (Sambrook *et al*., 1989).

V. siRNA Oligonucleotide Design

This section describes the process of identifying target sequences within a gene of interest and designing the corresponding oligonucleotides to generate the siRNA.

A. Selecting Target Sequences

1. Search for AA dimers within the coding sequence of your gene of interest. Identify the 19 nucleotides immediately downstream of the dimer. Do not select sequences within the 5' and 3' untranslated regions (UTRs) and regions near the start codon (within 75 bases) as these may be richer in regulatory protein binding sites (Elbashir e*t al.*, 2001). UTR-binding proteins and/or translation initiation complexes may interfere with binding of the RISC.
2. Calculate the GC content of the selected 19-base oligonucleotide sequence. The GC content should be between 30% and 70%; a GC content of approximately 50% is ideal.
3. Including the AA dimer in the candidate sequence, check the 21-base oligonucleotide for secondary structure and long base runs, which can interfere with proper annealing. Eliminate candidate sequences that display these characteristics.
4. Compare the remaining candidate sequences to an appropriate genome database to identify sequences that are specific for the gene of interest and show no significant homology to other genes. Candidate sequences that meet these criteria are potential siRNA target sites.

   To optimize gene silencing, we highly recommend that you test more than one siRNA target sequence for a gene. We provide enough RNAi-Ready pSIREN vector to perform 20 ligations, which allows you to screen for functional siRNA sequences within your gene of interest. You should test at least 4 siRNAs per gene. It may help to choose siRNA targets that are positioned all along the length of the gene sequence to reduce the chance of targeting a region that is either highly structured or bound by regulatory proteins.

   Note: You will need to design a gene-specific assay to test for the suppression of Gene X, if you have not already done so. See Section IV for additional information.

B. Designing Oligonucleotides

It is necessary to synthesize two complementary oligonucleotides (a top strand and a bottom strand) for each siRNA target site. Figure 4 illustrates the overall structure of the prototypical oligonucleotide sequences for use in pSIREN. The sequences of the oligonucleotides should include:

- A 5'-*Bam*H I restriction site overhang on the top strand and a 5'-*Eco*R I restriction site overhang on the bottom strand. These restric-

V. siRNA Oligonucleotide Design *continued*

tion sites will enable directional cloning of the annealed oligonucleotides into the RNAi-Ready pSIREN vector

- A purine (G or A) residue located just downstream of the *Bam*H I site on the top strand (RNA pol III prefers to initiate transcription with a purine)
- The 19-base oligonucleotide sense sequence (target sense sequence) of the siRNA target site
- A 7–9 nucleotide hairpin loop sequence (We typically use 5'-TTCAAGAGA- 3'; see Sui *et al.*, 2002; Lee *et al.*, 2002; Paddsion *et al.*, 2002; Brummelkamp *et al.*, 2002; and Paul *et al.*, 2002 for other effective loop sequences.)
- The 19-base oligonucleotide antisense sequence (target antisense sequence) of the siRNA target site; ensure proper orientation for correct formation of the hairpin structure (see Figure 2).
- A RNA pol III terminator sequence consisting of a 5–6 nucleotide poly(T) tract
- (Optional, but recommended) A unique restriction site immediately downstream of the terminator sequence for restriction digest analysis to confirm the presence of the cloned insert

A typical oligonucleotide has 5 bases for the restriction site at the 5' end, 19 bases of sense strand, 7–9 bases of hairpin loop, 19 bases of antisense strand, 6 bases of terminator, and 6 bases of a unique restriction site—resulting in an oligonucleotide of 62–64 bases. See Table II for examples of sense and antisense sequences designed for certain genes.

See Section IV for our recommendation to use PAGE-purified oligonucleotides. It is possible to clone without PAGE purification, but it is likely that the overall ligation efficiency and the number of correct clones will decrease due to the impact of incomplete oligonucleotide extensions. If the oligonucleotides are PAGE purified, order at the 200 nmol scale.

There is no need to order phosphorylated oligonucleotides. RNAi-Ready pSIREN Vectors have not been dephosphorylated after linearization; thus ligation will proceed smoothly using unphosphorylated oligonucleotides.

V. siRNA Oligonucleotide Design *continued*

```
                                                                                              Test RE
              BamH I ↓  Target sense sequence   Hairpin loop  Target antisense sequence  Terminator  site
Top strand     5'-GATCCGNNNNNNNNNNNNNNNNNNNTTCAAGAGANNNNNNNNNNNNNNNNNNNCTTTTTT-NNNNNN-G-3'
Bottom strand      3'-GCNNNNNNNNNNNNNNNNNNNAAGTTCTCTNNNNNNNNNNNNNNNNNNNGAAAAAA-NNNNNN-CTTAA-5'
                                                                                              EcoR I
```

Figure 4. siRNA oligonucleotide sequence design. Arrow denotes the purine residue required for RNA pol III to initiate transcription. The hairpin loop sequence shown is one of many functional loop sequences used to generate siRNAs. Termination is signaled using a poly(T) tract. Including a unique restriction site (Test RE site) allows confirmation of the cloned insert after the ligation and transformation reactions. 5' *Bam*H I and 3' *Eco*R I overhangs are necessary for directional cloning into RNAi-Ready pSIREN vectors. See Table II for examples of target sense and antisense sequences for certain genes.

TABLE II. EXAMPLES OF PUBLISHED TARGET SEQUENCES[a]

Gene	Target sequence[b]	Sense sequence	Antisense sequence	Reference
β-actin	AATGAAGATCAAGATCATTGC	TGAAGATCAAGATCATTGC	GCAATGATCTTGATCTTCA	Harborth e*t al.*, 2001
Bcr-abl	AAGCAGAGTTCAAAGCCCTT	GCAGAGTTCAAAGCCCTT	AAGGGCTTTGAACTCTGC	Scherr e*t al.*, 2002
hRad9	AAGTCTTTCCTGTCTGTCTTT	GTCTTTCCTGTCTGTCTTT	AAAGACAGACAGGAAAGAC	Hirai & Wang, 2002

[a] Sequences are shown for top strand oligo design. All sequences shown 5' to 3'. Bottom strand oligo design (not shown) is the complementary sequence to the top strand.
[b] Identified from gene coding sequence.

VI. Cloning into RNAi-Ready pSIREN Vectors

A. Annealing the Oligonucleotides

For convenience, Steps 3–6 can be done in a thermal cycler.

1. Resuspend each purified oligonucleotide in TE buffer to a concentration of 100 µM.
2. Mix the oligos for the top strand and the bottom strand at a 1:1 ratio. This will ultimately give 50 µM of ds oligo (assuming 100% theoretical annealing).
3. Heat the mixture to 95°C for 30 sec to remove all secondary structure.

   Note: Heating to 95°C ensures that the internal hairpin of each oligonucleotide is disrupted and promotes intermolecular annealing.
4. Heat at 72°C for 2 min.
5. Heat at 37°C for 2 min.
6. Heat at 25°C for 2 min.
7. Store on ice.

The annealed oligonucleotide is now ready for ligation into an RNAi-Ready pSIREN vector. Alternatively, the ds oligonucleotide can be stored at –20°C until ready to use.

B. Ligating ds Oligonucleotide into RNAi-Ready pSIREN

1. Dilute the annealed oligo (from Section VI.A.7) with TE buffer to obtain a concentration of 0.5 µM.

   Note: To ensure good ligation efficiency it is necessary to dilute the oligo so that it is only in moderate excess. Using an excess of the oligo will block ligation.
2. Assemble a ligation reaction for each experimental annealed oligonucleotide. Also set up ligations using the Luciferase siRNA and Negative Control siRNA Annealed Oligonucleotides. For each ligation, combine the following reagents in an Eppendorf tube:

2	µl	Linearized pSIREN vector (25 ng/µl)
1	µl	Diluted, annealed oligonucleotide (0.5 µM)*
1.5	µl	10X T4 DNA Ligase Buffer
0.5	µl	BSA (10 mg/ml)
9.5	µl	Nuclease-free H_2O
0.5	µl	T4 DNA ligase (400 U/µl)
15	µl	Total volume

   * Set up separate ligations using 1 µl of the Luciferase siRNA Annealed Oligonucleotide or 1 µl of the Negative Control Annealed Oligonucleotide. If desired, an additional control ligation can be assembled using 1 µl Nuclease-free H_2O instead of annealed oligonucleotide.
3. Incubate the reaction mixture for 3 hr at room temperature.

   Note: Do not let the ligation reaction go longer than 3 hr. If you are unable to immediately perform the transformation after this step, store the completed ligation at –20°C until ready to use.

VI. Cloning into RNAi-Ready pSIREN Vectors *continued*

C. Transforming BD Fusion-Blue™ Competent Cells with recombinant pSIREN

BD Fusion-Blue Competent Cells are an *E.coli* K-12 strain that provides high transformation efficiency. The strain carries recA and endA mutations that make it a good host for obtaining high yields of plasmid DNA. We routinely use this strain for all our siRNA cloning.

1. Thaw the required number of tubes of cells on ice for 10 min. Tap gently to ensure that the cells are suspended.
2. Add 2 µl of the ligation mixture (from Section VI.B.3) directly to 50 µl of cell suspension. Mix gently to ensure even distribution of the DNA solution.
3. Incubate the transformation mixture (DNA + cells) on ice for 30 min.
4. Heat the tubes for precisely 45 sec in a water bath at 42°C without shaking.
5. Remove the tubes from the water bath and place them directly on ice for 1–2 min.
6. Add 950 µl room-temperature SOC medium to each tube. Incubate at 37°C for 60 min while shaking at 250 rpm.
7. Plate 150 µl from each transformation on selective medium containing the appropriate concentration of antibiotic. Incubate at 37°C.

   Notes

   - Plating is accomplished by spreading cells on selective medium [e.g., LB agar + Ampicillin (50–100 µg/ml)]. See the Product Analysis Certificate that accompanies the RNAi-Ready pSIREN vector.
   - We have observed that recombinant pSIREN DNA usually generates smaller and slower-growing colonies.

8. Inoculate a small-scale liquid culture with a single, well-isolated colony. We recommend you set up 4–8 such cultures to ensure you obtain at least one positive clone. After overnight incubation, isolate plasmid DNA using any standard method. For small-scale purification (≤20 µg plasmid DNA), we recommend our NucleoSpin Multi-8 Plus Plasmid Kit (Cat. No. 635976).

VI. Cloning into RNAi-Ready pSIREN Vectors *continued*

9. Identify the desired recombinant plasmid by restriction analysis using the unique restriction site within the siRNA oligonucleotide sequence. If desired, verify your insert by sequencing.

   Note: Since there is always a chance for mutations in the oligo due to synthesis errors, we strongly recommend that you sequence at least two clones to verify the correct oligo sequence. Because hairpin sequences are difficult to sequence, inform your sequencing facility so that sequencing conditions can be adjusted accordingly.

10. Once a positive clone has been identified, inoculate a large-scale liquid culture to prepare greater quantities of your recombinant pSIREN vector. To ensure optimal purity of the DNA, isolate all plasmids for transfection using a NucleoBond Plasmid Maxi EF Kit (Cat. No. 635953) or by banding on a CsCl gradient (Sambrook *et al*., 1989).

VII. Transfection of Recombinant pSIREN Vectors

The transfection protocol included in this User Manual is intended for both the screening for functional siRNA constructs and gene silencing experiments using transfection. If you will perform experiments using viral delivery, first use this transfection procedure to screen constructs, then proceed to the secondary User Manuals included with your BD Knockout RNAi System. The secondary User Manuals describe the protocols for viral packaging and infection using pSIREN constructs. If your target cells cannot be transfected, then viral delivery should be tried for both functional siRNA screening and gene silencing experiments.

For further information on cell culture techniques, see Freshney (1993).

The efficiency of a mammalian transfection procedure is primarily dependent on the host cell line. Therefore, when working with a cell line for the first time, we recommend you compare the efficiencies of several transfection protocols. After choosing a method of transfection, optimize cell density (usually 60–80% confluency), the amount and purity of the DNA, media conditions, and transfection time.

If a transfection method is already established for your cell line model, proceed with those conditions. It is important to keep optimized parameters constant to obtain reproducible results.

For our transfections, we have been successful using the BD CalPhos™ Mammalian Transfection Kit (Cat. No. 631312) and the BD CLONfectin™ Transfection Reagent (Cat. No. 631301).

The following protocol is designed for use with BD CLONfectin Transfection Reagent using adherent cultures in 6-well tissue-culture plates. If you are using other vessels, adjust the components in proportion to the surface area of your plate/flask. See the BD CLONfectin User Manual (PT3005-1), Appendix B for culture plate conversions. **Perform all steps in a sterile tissue culture hood.**

1. Plate cells two days before the transfection experiment; they should be 60–70% confluent on the day of transfection. We generally plate 1–4 x 10^5 cells/well.
2. Incubate plates at 37°C in a CO_2 incubator for 24–48 hr.
3. On the day of the transfection, prepare fresh liposome solution:
   a. Warm HEPES-Buffered Saline (HBS) to 45–55°C.
   b. Add 90 µl of HBS dropwise to a 100-µg aliquot of BD CLONfectin Reagent stock to make a final concentration of 1 µg/µl.
   c. Immediately vortex gently.
   d. Place on ice.

VII. Transfection of Recombinant pSIREN Vectors *cont.*

4. For each transfection, prepare solution A and solution B in separate, sterile polystyrene tubes.

   Solution A:

2–4 µg	plasmid DNA
100 µl	Serum-free medium

   Solution B:

2–8 µg	BD CLONfectin Reagent (1 µg/µl in HBS)
100 µl	Serum-free medium

   Notes:
   - A 1:1 (w/w) ratio of DNA:BD CLONfectin Reagent works best for many cell types.
   - To reduce variability when transfecting multiple wells with the same plasmid DNA, prepare adequate volumes of solutions A and B for all wells.
   - You may store unused BD CLONfectin Reagent at 4°C up to one week.
5. Combine solutions A and B into one tube and mix gently.
6. Incubate the transfection solution at room temperature for 10–30 min.
7. Add 1.8 ml of serum-free medium to each tube containing the BD CLONfectin Reagent/DNA solution and mix gently.
8. Remove the medium from the cultures to be transfected, wash with PBS, and gently apply the BD CLONfectin Reagent/DNA/media solution.

   Notes:
   - Washing the cells with serum-free medium before applying the BD CLONfectin/DNA solution is not necessary. Any serum present will be very dilute (<1%) and will not affect transfections using BD CLONfectin Reagent. You may add serum to a final concentration of 2.5% to the BD CLONfectin Reagent/DNA-containing medium at this point to improve cell viability.
   - Do not add antibacterial or antifungal agents to the medium during transfection.
9. Gently rock plates back and forth to distribute transfection solution evenly. Do not rotate plates as doing so will concentrate transfection precipitate in the center of the well.
10. Incubate plates at 37°C for 4–6 hr in a CO_2 incubator.
11. Remove BD CLONfectin Reagent/DNA-containing medium and wash cells with medium or 1X PBS. Prewarm media or PBS to 37°C.
12. Apply 2 ml of fresh complete growth medium and incubate at 37°C until cells are needed for assay.
13. Assay for transient gene suppression 48 hr post-transfection.

    Note: If you have transfected a pSIREN-RetroQ construct, you can start selection for stable transformants 48–72 hr post-transfection.