Chapter 1

Construction of Modular Lentiviral Vectors for Effective Gene Expression and Knockdown

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Abstract

Elucidating gene function is heavily reliant on the ability to modulate gene expression in biological model systems. Although transient expression systems can provide useful information about the biological outcome resulting from short-term gene overexpression or silencing, methods providing stable integration of desired expression constructs (cDNA or RNA interference) are often preferred for functional studies. To this end, lentiviral vectors offer the ability to deliver long-term and regulated gene expression to mammalian cells, including the expression of gene targeting small hairpin RNAs (shRNAmirs). Unfortunately, constructing vectors containing the desired combination of cDNAs, markers, and shRNAmirs can be cumbersome and time-consuming if using traditional sequence based restriction enzyme and ligation-dependent methods. Here we describe the use of a recombination based Gateway cloning strategy to rapidly and efficiently produce recombinant lentiviral vectors for the expression of one or more cDNAs with or without simultaneous shRNAmir expression. Additionally, we describe a luciferase-based approach to rapidly triage shRNAs for knockdown efficacy and specificity without the need to create stable shRNAmir expressing cells.

Key words Gateway cloning, Lentiviral vectors, Short hairpin RNA (shRNA), Dual-luciferase reporter, shRNA triage, Design, Delivery, Lentivirus

1 Introduction

The last few decades have been revolutionary in the extensive amount of genomic data that has become available to researchers for the study of gene expression and function. This wealth of knowledge and other technological advances have made it possible to interrogate the biological role of genes in both diseased and normal tissues. Using gene sequence information, researchers have been able to introduce DNA into in vitro or in vivo study systems for the overexpression or suppression of genes of interest. For cell culture based studies, often delivery of these elements has relied on transient expression from plasmid-based technology, using transfection or electroporation. Unfortunately, this type of delivery method does not provide long-term gene expression or gene

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knockdown. Furthermore, some cell types are not amenable to DNA uptake in this manner. Fortunately, retroviral and lentiviral vectors provide the means to yield stable integration of genetic components for the sustained expression or knockdown of genes of interest [1–3]. Lentiviruses additionally provide the ability to transduce dividing and non-dividing cells [4]. For their utility in producing stable vector integration into a host genome, retroviral and lentiviral constructs have been used extensively in the functional analysis of genes. As such, several vendors now provide retroviral and lentiviral constructs for cDNA overexpression or the expression of RNA interference (RNAi) for gene knockdown (reviewed in refs. [2, 5]).

Constructing lentiviral vectors for the expression of cDNA or RNAi using restriction and ligation strategies can be a constraining and time-consuming undertaking. Recently, the cloning process has been simplified by the use of ligation-independent methods for vector construction [6–12]. To avoid the use of traditional cumbersome methods of subcloning, one crucial step has been the implementation of recombination-based cloning systems such as Gateway technologies (Invitrogen). Gateway cloning technology is based on the site-specific recombination properties of bacteriophage λ [12, 13]. This phage inserts its DNA into the bacterial genome in between specific DNA attachment sites termed attPx (*p*hage *att*achment site) and attBx (*b*acterial *att*achment site), creating attLx (left end of prophage) and attRx (right end of prophage) sites. This phenomenon has been harnessed and commercialized as Gateway® cloning technology to allow for the efficient and precise transfer of desired DNA sequences from one plasmid to another by site-specific recombination. Using LR recombination (between attL and attR sites), recombinant expression plasmids can be created by transferring a desired attL flanked DNA fragment from an entry plasmid(s) into a destination plasmid containing an attR flanked bacterial lethal gene, *ccdB* [14]. The *ccdB* cassette ensures any non-recombinant destination plasmids or recombinant entry plasmids that carry it would be negatively selected against. Furthermore, entry plasmids are typically kanamycin resistant, whereas destination plasmids are ampicillin resistant, thus allowing for positive selection of the desired recombinant expression plasmid [15]. The specificity of Gateway cloning allows for the rapid construction of the plasmids containing cDNA and/or RNAi elements that are inserted unidirectionally by virtue of variants made to the attL and attR sequences. Increasing the number of attL/attR variants has expanded the utility of this system to permit directional, ordered cloning of multiple DNA inserts into an expression plasmid [16].

In recent years, RNAi has been used as a powerful investigative tool to elucidate the function of nearly any gene whose sequence is

available. The simplest approach of delivering gene silencing RNAi is the transfection of short interfering RNA oligonucleotides (siRNA) [17, 18]. This can be an effective way to study the short-term effects of gene expression knockdown, although potential off-target effects due to initial high cytosolic siRNA concentrations occur (reviewed in ref. [19]). Additionally, knockdown is transient since siRNA concentration is diluted after several rounds of cell division. Alternatively, vector driven expression of short hairpin RNAs (shRNAs) may be used to create stable knockdown [19, 20]. shRNA expression can be driven from RNA Polymerase II or III promoters. While Pol III driven shRNA expression from promoters such as U6 and H1 were initially used [21, 22], the high levels of shRNA expression delivered can saturate the endogenous shRNA processing machinery [23–26]. Moreover, Pol III driven transcripts are less amenable to driving tissue-specific or inducible expression (reviewed in ref. [27], with exceptions noted). Newer Pol II driven shRNA vectors, mimic the structure of microRNAs (miRNAs), with shRNA sequences typically embedded in the human based miRNA-30 element (shRNAmir) [28–30]. Pol II driven shRNA expression has many advantages over the Pol III equivalent including the feasibility of inducible or tissue-specific expression and simultaneous expression of several shRNAmirs from a single polycistronic transcript [27, 29, 31]. While vectors for the stable shRNAmir expression are commercially available they are typically costly and are limited to a few selectable markers. In the construction of these vectors, shRNA target sites are selected using algorithms designed to predict target sequences that should produce effectual knockdown [32–34]. Moreover, because the precise sequence requirements for effective shRNA processing and targeting are still incompletely understood, commercially available targeting constructs are not guaranteed to successfully suppress gene expression [35].

Here we describe a novel method for the design and rapid triage of shRNA without the need to create stable shRNAmir expressing cell lines. Using a luciferase-based approach, shRNA efficacy and specificity can be assessed in a medium-throughput fashion to yield candidates for functional knockdown in vitro (Fig. 1). Furthermore, the shRNAmir expression vectors utilized in the triaging process are compatible with the Gateway cloning system. Using Multisite Gateway technology, we additionally describe techniques to rapidly construct and use lentiviral expression vectors that are capable of delivering one or more cDNAs along with simultaneous shRNAmir expression (Fig. 2). This system was constructed to facilitate efficient and flexible cloning of various elements (cDNA, markers, and shRNAmirs) into lentiviral vectors for desired combinations of gene expression and knockdown [36].

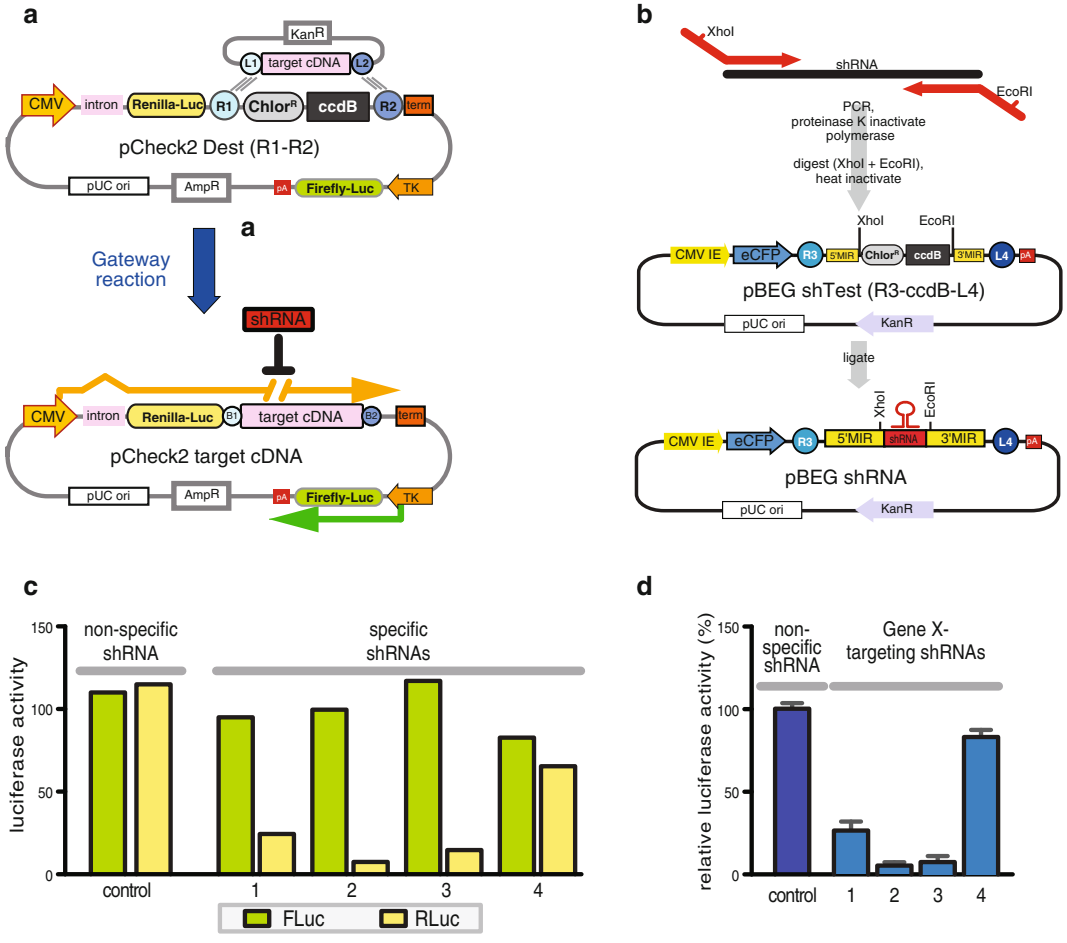

Fig. 1 Rapid triage of novel shRNAs. (**a**) Target cDNAs are cloned into pCheck2 Dest (R1–R2) through Gateway recombination between attR1–attR2 sites and attL1–attL2 compatible with all standard cDNA pEntry plasmids (attL1–attL2). The resulting plasmid produces two transcripts: a CMV-driven transcript (*yellow arrow*) encoding Renilla luciferase and a non-translated cDNA target and a TK-driven transcript (*green arrow*) encoding firefly luciferase to serve as an internal transfection control. (**b**) General method for the PCR amplification of novel shRNAs from a ~100 bp oligonucleotide core (e.g., shRNA2) with two universal primers (*red arrows*). After high fidelity PCR, the polymerase is inactivated by proteinase K treatment; the proteinase K is heat inactivated and then the PCR product is digested with XhoI/EcoRI. The restriction enzymes are subsequently heat inactivated and the fragment is cloned into the corresponding sites of pBEGshTest (R3-ccdB-L4) to create an attR3-attL4 based Entry vector pBEG shRNA. (**c**) Firefly and Renilla luciferase activities are measured sequentially for lysates derived from pCheck2 and pBEG shRNA coexpressing cells. Different shRNAs are compared by normalizing for transfection efficiency via Firefly Luciferase and then for specific knockdown by assessing Renilla luciferase levels. (**d**) This data is normalized to the luciferases observed for the nonspecific shRNA control

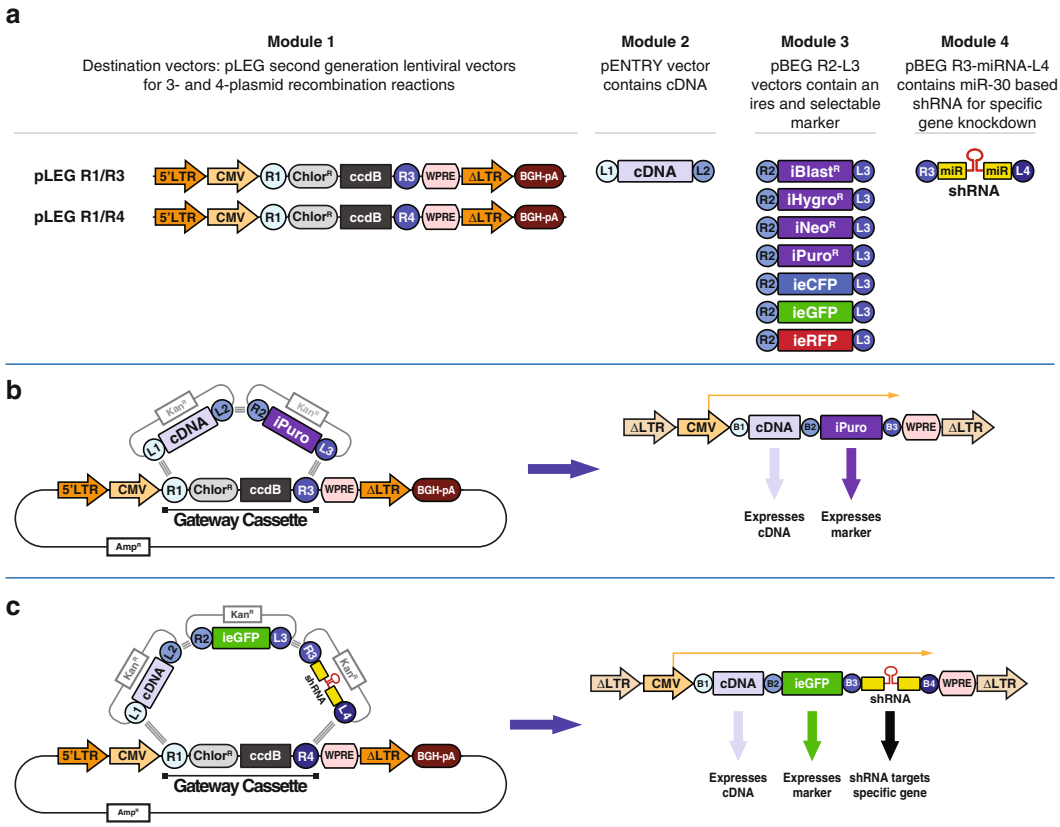

Fig. 2 A modular lentiviral vector system. (**a**) This modular system consists of a lentiviral vector backbone (Module 1), a cDNA contained in a standard ENTRY vector (Module 2), genetic markers or florescent proteins encoded downstream of internal ribosome entry sequences and are flanked by attR2 and attL3 sites contained in a pBEG vector (Module 3), and an optional miR30-embedded shRNA flanked by attR3 and attL4 sites (Module 4). (**b**) A three-way Gateway LR recombination reaction to insert a cDNA and marker of choice into the pLEG R1–R3 Destination vector. (**c**) A four-way Gateway LR Recombination reaction to insert a cDNA, marker, and shRNA of choice into the pLEG R1–R4 Destination vector

2 Materials

Prepare all solutions using ultrapure water and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise).

2.1 Design and Cloning of shRNA into pBEG Expression Vector

1. Primers for 3 primer PCR:
   (a) 10 μM shRNA template.
   (b) 10 μM universal primers:
   Fwd: 5′-CACCCTCGAGAAGGTATATTGCTGTTGACAGTGAG-3′.
   Rev: 5′-CCCCTTGAATTCCGAGGCAGTAGGCA-3′.

2. Phusion® High-Fidelity DNA Polymerase, or other proofreading polymerase with comparable low error rate (New England BioLabs).
3. 5× HF Buffer (provided with Phusion polymerase).
4. DMSO.
5. 10 mM dNTP mix.
6. PCR plate or tubes.
7. Thermocycler.
8. 20 mg/mL Proteinase K.
9. XhoI and EcoRI restriction enzymes.
10. Agarose.
11. TAE buffer: 40 mM Tris–acetate and 1 mM EDTA.
12. pBEG shTest plasmid, or other shRNAmir expression plasmid with XhoI/EcoRI sites flanked by a miRNA-30 cassette (*see* **Note 1**).
13. Gel purification kit.
14. T4 DNA Ligase (New England BioLabs).
15. 10× T4 DNA Ligase Reaction Buffer.
16. Chemically competent, ccdB-sensitive bacteria (e.g., DH5α, DH10B).
17. SOC medium: 2% w/v tryptone, 0.5% w/v yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, and 20 mM glucose.
18. LB agar plates with 50 μg/mL kanamycin.
19. Miniprep plasmid DNA isolation kit.

2.2 Luciferase Assay Triaging of shRNAs

1. Target cDNA sequence flanked with L1–L2 Gateway recombination sites in an entry plasmid (*see* **Note 2**).
2. pCheck2 Dest (R1–R2) plasmid (Addgene #48955) (*see* **Note 2**).
3. LR Clonase II (Invitrogen).
4. 293T cells.
5. 24-well cell culture plates.
6. Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin.
7. 1.5 mL microtubes.
8. Opti-MEM (Invitrogen).
9. 1 mg/mL polyethylenimine (PEI).
10. Dual-Luciferase® Reporter Assay System (Promega).

11. Phosphate buffered saline (PBS).
12. Luminometer (single-sample or a multi-sample/plate-reader).
13. Luminometer compatible tubes or multi-well plates.

2.3 Construction of Recombinant Lentiviral Vectors

1. Entry vectors as required by your application:
   (a) 2 component lentiviral vector: L1–L2 entry plasmid (e.g., cDNA of choice), R2–L3 entry plasmid (e.g., marker of choice).
   (b) 3 component lentiviral vector: L1–L2 entry plasmid (e.g., cDNA of choice), R2–L3 entry plasmid (e.g., marker of choice).
2. Destination vector: pLEG (R1–R3) or pLEG (R1–R4).
3. LR Clonase II Plus (Invitrogen).
4. Tris–EDTA (TE) pH 8.0: 10 mM Tris–Cl and 1 mM EDTA.
5. 20 mg/mL proteinase K.
6. Chemically competent, ccdB-sensitive bacteria.
7. SOC medium.
8. LB agar plates with 50 μg/mL carbenicillin.
9. Miniprep plasmid isolation kit.
10. Glycerol.
11. Midiprep plasmid isolation kit.

2.4 Production of Lentivirus

1. 293T cells.
2. 10 cm cell culture plates.
3. Lentiviral vector, Gag-Pol plasmid (e.g., pAX2), and VSV-G plasmid (e.g., pMDG).
4. Opti-MEM (Invitrogen).
5. 1 mg/mL polyethylenimine (PEI).
6. 0.45 μM syringe filter.

2.5 Stable Transduction of Cells with Lentivirus

1. Viral supernatant from Subheading 2.4.
2. Cells to be transduced.
3. Growth medium for the cells.
4. 10 mg/mL stock polybrene (Hexadimethrine bromide, Sigma H9268).
5. Appropriate agent for drug selection if lentiviral vector contains a drug-resistance gene.
6. Materials for confirmation of lentiviral construct expression (e.g., Western blot or real-time PCR equipment).

3 Methods

3.1 Design and Cloning of shRNA into pBEG shRNA Expression Vector

1. Select potential target sites in your gene of interest by finding published siRNA or shRNA sequences that have been functionally validated. If no sequences are found in the published literature, consult online RNAi database(s) for deposited shRNA sequences or design the shRNA anew using online design tools (*see* **Note 3**). Select at least two different targeting sequences to control for the possible effects due to off-target knockdown.
2. Select a control shRNA. Design either a non-targeting scramble shRNA or an shRNA targeting another gene, preferably in a different species. BLAST the shRNA sequence to ensure there are no additional homologies to the chosen sequence.
3. Format the target sequences in an shRNA template as follows: tgctgttgacagtgagCG-**X**(**N18–22**—*TAGTGAAGCCACA-GATGTA*—**N′18-22**)**X′**-tgcctactgcctcgaat (*see* **Note 4**). Order this DNA oligonucleotide for use in the next step.
4. To add the flanking XhoI/EcoRI sites to the shRNA template, perform a three primer PCR reaction between the shRNA template and the two universal primers (*see* **Note 5**). Prepare a PCR master mix including the following components per shRNA template to be amplified:

   5.0 μL HF buffer.

   1.0 μL 10 μM Fwd universal primer.

   1.0 μL10 μM Rev universal primer.

   0.5 μL of 10 mM dNTP stock.

   15.0 μL ddH_2O.

   1.25 μL DMSO.

   0.25 μL (0.5 U) Phusion polymerase (add last).

   Add 24 μL of the master mix to 1.0 μL of each shRNA template (dilute shRNA template DNA to 10 μM first). Perform PCR using the following thermal cycler program:

   Step 1. 98 °C × 2 min.

   Step 2. 98 °C × 10 s.

   Step 3. 60 °C × 30 s.

   Step 4. 72 °C × 1 min.

   Repeat **steps 2–4** for a total of 30 cycles.

   Step 5. 72 °C × 10 min.

   Step 6. Cool to 4 °C.

5. Inactivate Phusion polymerase by adding 1 μL of proteinase K to each PCR reaction and incubate in the thermal cycler with the following program:

   37 °C × 30 min.

   95 °C × 30 min (to inactivate the proteinase K).

6. Digest the 10 μL of the PCR product with XhoI/EcoRI in a 20 μL total reaction volume for 1 h at 37 °C. Incubate the digests at 85 °C for 20 min to heat inactivate the restriction enzymes.
7. Digest 1–5 μg of pBEG shTest plasmid with XhoI/EcoRI for 2–4 h. Run the digest on a 0.8% agarose-TAE gel for 40 min at a constant voltage of 120 V. Gel purify the 4333 bp fragment in a final elution volume of 30 μL.
8. Ligate the digested shRNA templates to the gel purified pBEG shTest backbone in a 20 μL total reaction volume from 4 h to overnight at room temperature. If performing the ligation using T4 DNA Ligase, prepare the following reaction recipe per ligation reaction:

   3.0 μL digested shRNA template.

   3.0 μL gel purified shTest backbone.

   11.0 ddH_2O.

   2.0 μL 10× Ligation Buffer.

   1.0 μL Ligase.

9. Transform 2–4 μL of the ligation reaction into 50 μL of chemically competent ccdB-sensitive bacteria in a 1.5 mL microtube on ice for 30 min.
10. Add 700 μL of antibiotic-free SOC medium to the microtube and grow the bacteria in a 37 °C shaker before plating on kanamycin-containing LB agar plates.
11. Incubate plates overnight at 37 °C and inoculate colonies into LB-kanamycin for overnight growth in a 37 °C shaker.
12. Miniprep the pBEG shTest R3-shRNA-L4 DNA using a miniprep kit. Use a protocol for the isolation of a low-copy plasmid (*see* **Note 6**).

3.2 Luciferase Assay Triaging of shRNAs

1. Clone the intended target cDNA into the pCheck2 Dest (R1–R2) vector (*see* **Note 2**) and midiprep the DNA for use in the luciferase assays.
2. The day before transfection (Day 1), seed 293T cells into 24-well plates at 5×10^4 cells/well (*see* **Note 7**). Seed 3 wells as technical replicates for each shRNA to be tested. Allow the cells to adhere overnight.

3. Prepare the pBEG shTest and pCheck2 DNA for co-transfection of the 293T cells (Day 2). Standardize pBEG shTest and pCheck2 DNA to 100 ng/uL (*see* **Note 8**). For each well to be transfected, combine 0.46 μg of pBEG shTest and 0.20 μg of pCheck2 DNA in a microtube (*see* **Note 9**). In a sterile cell culture hood, add 100 μL of Opti-MEM and 2 μL of PEI to each tube (*see* **Note 10**). Mix by inverting. DO NOT vortex. Incubate the DNA–Opti-MEM–PEI mix for 30 min at room temperature.
4. Before transfecting, replace the medium on the 293T cells with 500 μL/well of fresh DMEM. Add the transfection mix to the cells dropwise. Incubate the cells at 37 °C overnight.
5. The morning following transfection (Day 3), remove the transfection medium and replace with 500 μL/well of fresh DMEM.
6. About 48 h post-transfection (Day 4), prepare the reagents for the luciferase assay (1× Passive Lysis Buffer, LAR II, and Stop & Glo®) as instructed by the Promega Dual-Luciferase Assay System protocol (*see* **Note 11**). Prepare enough of each reagent to use 100 μL per replicate/well. Make sure all the reagents have reached room temperature before use.
7. Rinse the 293T cells with 500 μL of 1× PBS (*see* **Note 12**). Remove PBS. Add 100 μL of 1× Passive Lysis Buffer to each well and shake/rock the plate gently at room temperature for 30 min.
8. Read luminescence using either a single-sample luminometer or a multi-sample/plate-reading luminometer (*see* **Note 13**). If using a manual luminometer or a luminometer fitted with one reagent injector use the following instructions:
   (a) Predispense 100 μL of the LAR II reagent into the appropriate number of luminometer compatible tubes required to assay all of the samples.
   (b) Pipette 5 μL of 293T lysate into the LAR II reagent and pipette up and down 10 times to mix (*see* **Note 14**). DO NOT vortex.
   (c) Count 2 s (or program luminometer to delay 2 s) to allow the sample to equalize before measuring Firefly luciferase luminescence with a read time of 10 s in the luminometer. If the luminometer is not connected to a computer or a printer, manually record the luminescence measurement.
   (d) Remove the sample from the instrument and pipette 100 μL of Stop & Glo® reagent (or use reagent injector to dispense this) into the tube. Vortex for 2 s then count 2 s before measuring the Renilla luciferase luminescence with a read time of 10 s in the luminometer. Record the luminescence measurement.
   (e) Repeat **steps b–d** for the remaining samples.

9. To assess shRNA knockdown efficiency, divide the Renilla luciferase activity measurement by the Firefly luciferase activity measurement for each sample and normalize these ratios to the Renilla–Firefly ratio for the non-targeting control shRNA.

3.3 Construction of Recombinant Lentiviral Vectors

1. For your application, decide upon the combination of components (cDNA and marker, with or without shRNAmir) you will need to express with the lentiviral construct (*see* **Note 15**):
   (a) For a 2 component lentiviral vector you require three plasmids for the LR reaction (three-way recombination): (1) An entry plasmid with an L1–L2 flanked cDNA sequence, (2) An entry plasmid with an R2–L3 flanked marker (drug selection or fluorophore), and (3) the lentiviral destination plasmid pLEG R1–R3.
   (b) If shRNAmir expression is desired, a 3 component lentiviral vector can be constructed for which you require four plasmids for the LR reaction (four-way recombination): (1) An entry plasmid with an L1–L2 flanked cDNA sequence, (2) An entry plasmid with an R2–L3 flanked marker (drug selection or fluorophore), (3) An entry plasmid with an R3–L4 flanked shRNAmir (e.g., pBEG shTest), and (4) the lentiviral destination plasmid pLEG R1–R4.
2. Prior to use in Gateway LR recombination reactions, dilute entry plasmid DNA to 10 fmol/μL and destination plasmid DNA to 20 fmol/μL in ddH_2O.
3. To set up the LR recombination reactions, in a microtube add 0.5 μL of each entry plasmid and 0.5 μL of the destination plasmid to 0.5 μL of LR Clonase II Plus, and make up the reaction volume to 5 μL with TE. Incubate at room temperature for 16–24 h.
4. Add 1 μL of Proteinase K to the reaction and incubate at 37 °C for 20–30 min to terminate the reaction.
5. Transform the entire reaction into 50–100 μL of chemically competent, ccdB-sensitive bacteria in a 1.5 mL microtube on ice for 30 min (*see* **Note 16**).
6. Add 700 μL of antibiotic-free SOC medium to the microtube and grow the bacteria in a 37 °C shaker for 1 h. Plate the bacteria on carbenicillin LB agar plates.
7. Miniprep at least six colonies, saving an aliquot of bacteria from each prep to freeze at −80 °C in 15% glycerol. The frozen stock will be used to inoculate a midiprep culture once the miniprep DNA has been screened.
8. Midiprep the correct recombinant plasmid for the production of lentivirus.

3.4 Production of Lentivirus Vector Particles

1. On Day 1, plate 5×10^6 293T cells in a 10 cm plate in DMEM. Allow cells to adhere overnight.
2. On Day 2, replace the medium on the 293T cells with 9 mL of fresh DMEM.
3. Aliquot the lentiviral vector and the packaging vectors into a 1.5 mL microtube in the following amounts: 8 μg of lentiviral vector, 5.2 μg of Gag-Pol plasmid (e.g., pAX2), and 2.8 μg of a VSV-G plasmid (e.g., pMDG).
4. In a sterile cell culture hood, add 550 μL of Opti-MEM to the DNA followed by the dropwise addition of 43 μL PEI. Invert to mix. Incubate the DNA–Opti-MEM–PEI mixture for 30 min at room temperature.
5. Add the transfection mixture to the plate of 293T cells dropwise. Incubate the cells overnight at 37 °C.
6. On Day 3, remove the transfection medium and replace with 7 mL of fresh DMEM.
7. On Day 4, harvest the lentivirus vector particles by collecting the medium off the cells and filtering the harvested medium through a 0.45 μM filter. Use the harvested medium immediately for transduction of recipient cells or store at 4 °C for up to 2 weeks. For long-term storage, keep at −80 °C.

3.5 Stable Transduction of Cells with Lentivirus Vector Particles

1. On Day 1, seed 5×10^5 of the cells to be transduced in a 10 cm plate. Let the cells adhere overnight (*see* **Note 17**).
2. On Day 2, change the medium on the cells for 5 mL of culture medium containing polybrene at 10 mg/mL and 5 mL of viral supernatant. Transduce the cells overnight at the appropriate growth temperature.
3. On Day 3, replace the medium on the cells with the regular growth medium.
4. On Day 5, replace the medium on the cells with growth medium containing the appropriate selection drug. Culture cells as needed in selection drug.
5. Confirm expression (e.g., shRNA knockdown) of the lentiviral construct with Western blot or real-time PCR analysis (Fig. 3).

4 Notes

1. We recommend the use of the pBEG shTest plasmid for shRNA triaging purposes since its efficacy has been validated within this experimental design and it will make subsequent cloning into lentiviral vectors much simpler. (Available through Addgene.)

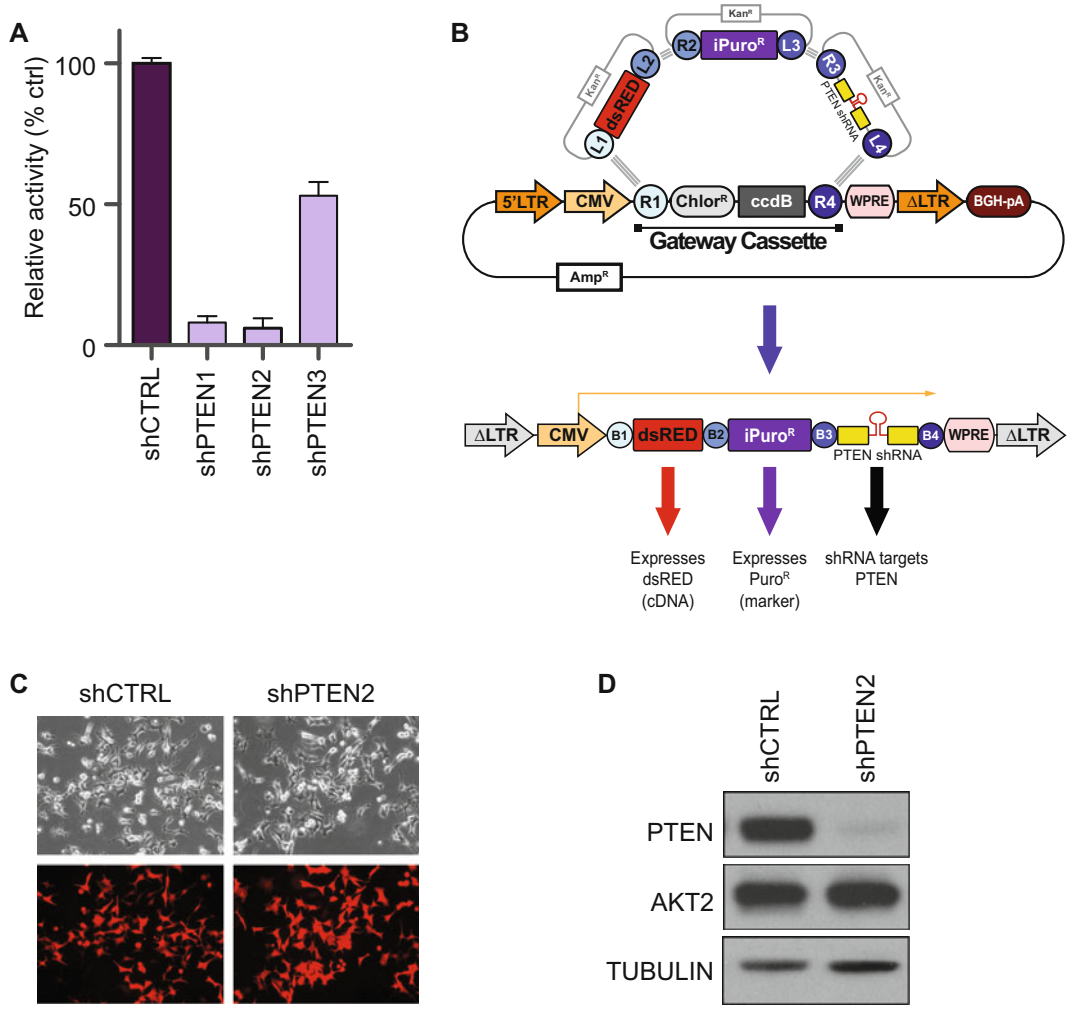

Fig. 3 *PTEN* shRNA triage, Gateway cloning, and in vitro validation. (**a**) Luciferase assay triage of PTEN specific shRNAs. (**b**) A Multisite Gateway LR recombination reaction to create a lentiviral vector expressing the dsRed fluorophore, a puromycin resistance gene, and a PTEN targeting shRNA (pLEG-dsRed-iPuro-shPTEN2). (**c**) dsRed expression in WM1617 melanoma cells infected with pLEG-dsRed-iPuro-shRNA lentivirus (post-puromycin selection). (**d**) Western blot analysis of PTEN specific knockdown in WM1617 pLEG-dsRed-iPuro-shRNA cells

2. If possible, obtain a plasmid that contains the target cDNA sequence flanked by attL1–attL2 Gateway recombination sites. The cDNA sequence can then be inserted downstream of Renilla luciferase in between the pCheck2 R1-R2 sites using a two-plasmid Gateway LR recombination reaction. For this Gateway recombination reaction use 10 fmol entry plasmid, 20 fmol pCheck2 R1–R2, and 1 μL LR Clonase II in a total of 5 μL and follow the rest of the instructions as laid out in Subheading 3.3. For commercially available cDNAs or ORFs

in attL1–attL2 entry plasmids, consult plasmid repositories at https://dnasu.org/DNASU/Home.do, http://www.addgene.org, or http://www.genecopoeia.com. If such a plasmid does not exist, the target cDNA should first be cloned into the attL1–attL2 sites of the Gateway compatible pENTR1A or pENTR/D-TOPO plasmids (Life Technologies) via restriction enzyme site cloning or TOPO cloning, respectively.

3. Database of pre-constructed shRNA target sites are available at The RNAi Consortium http://www.broadinstitute.org/rnai/public/ or RNAi Codex http://cancan.cshl.edu/cgi-bin/Codex/Codex.cgi. To design shRNAs anew consult websites that offer algorithms for shRNA target site selection such as the one available here http://www.genelink.com/sirna/shrnai.asp.

4. Designing the shRNA template as follows: 5′-tgctgttgacagtgagCG-**X**(**N18-22**–*TAGTGAAGCCACAGATGTA*-**N'18-22**)**X'**-tgcctactgcctcgaat (Fig. 4). Here, the portion in italics represents the constant 19 bp "loop sequence" flanked by 19–23 bp sense (**X**-**N**) and antisense (**N′**-**X′**) target sequences (loop structure based on [37, 38]). Bolded nucleotides vary depending on the target sequence. The **X′** represents the last 3′ nucleotide of the antisense sequence and should compliment the intended target sequence because it is the antisense strand that binds to the target mRNA to elicit knockdown. The **X** represents the first 5′ nucleotide of the sense sequence that should be changed to be uncomplimentary to whatever nucleotide replaces the **X′** in the antisense sequence. If **X′** is an A or a T, change **X** to a C and if **X′** is a C or a G, change **X** to an A. This creates a bubble that is required for the proper endogenous processing of the shRNA by Dicer [38]. The small case sequences share homology with the universal primers to be used for PCR. For example to target human *PTEN* the following shRNA template was used: 5′-tgctgttgacagtgagCG-**A(AGGAACAATATT-GATGATGTA***TAGTGAAGCCACAGATGTA***TACAT-CATCAATATTGTTCCT)G**-tgcctactgcctcggaat. Note the A(…)G mismatch outside the parenthesis The G represents the antisense sequence that will target a cytosine in the *PTEN* mRNA (CAGGAACAATATTGATGATGTA).

5. The universal primers, (Fwd) 5′-CACC**CTCGAG**AAG GTATATtgctgttgacagtgag-3′ and (Rev) 5′-CCCCTT**GA attcc**gaggcagtaggca-3′, add flanking XhoI/EcoRI sites for subsequent cloning into pBEG shTest (primers based on those used by Chang et al. [37]). Bolded are the XhoI/EcoRI sites and small case are the portions of the primers homologous to the shRNA template. The expected PCR product has a CCAC at the 5′ end so it may be cloned into a TOPO vector if major problems are encountered when trying to clone the PCR product directly into the pBEG shTest vector.

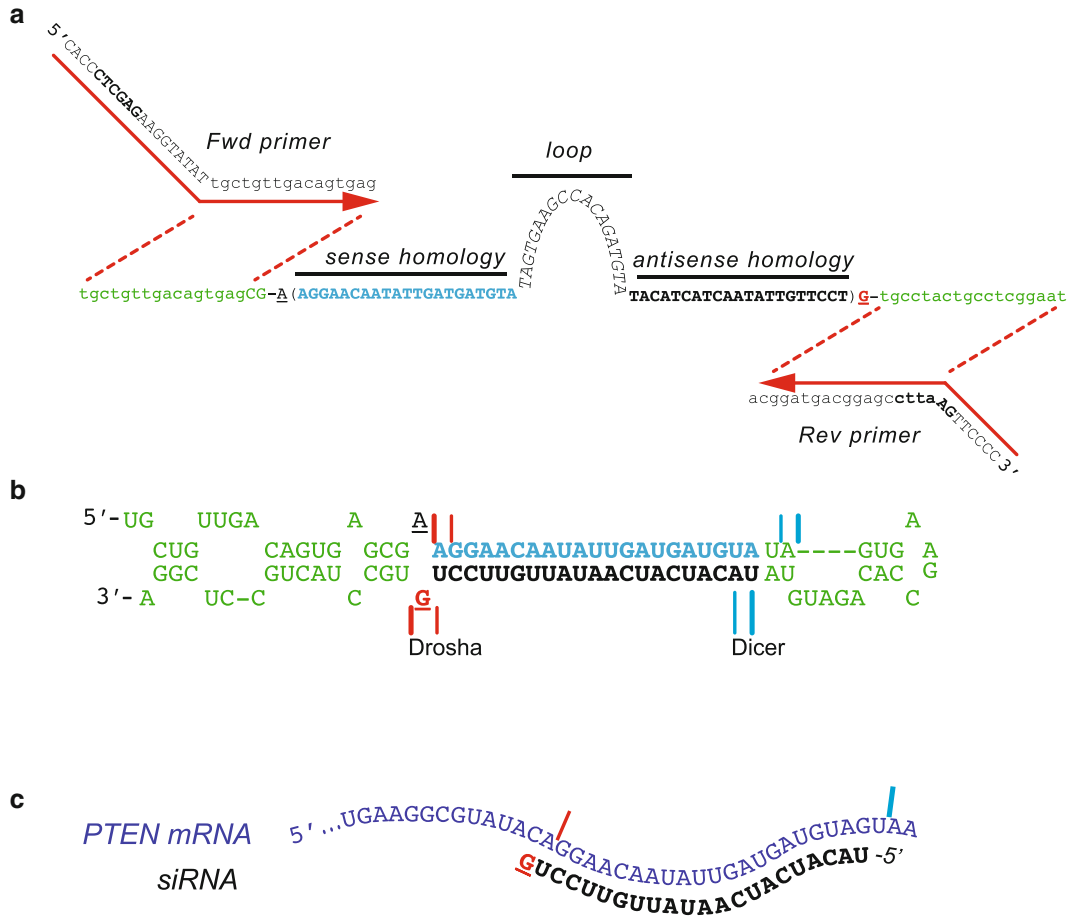

Fig. 4 shRNA design. (**a**) PTEN shRNA template DNA is depicted along with the forward and reverse primers for cloning. *Bolded* are gene specific sequences with *blue* and *black* being the sense and antisense sequences respectively. Sequences in *green* represent vector derived sequences not homologous to the target mRNA. *Lower case nucleotides* represent sequences the PCR primers bind to. *Underlined bases* are mismatched with the 3′ most underlined sequence being complementary to the target RNA (here a G). (**b**) Predicted structure of the shRNA and flanking sequence along with the predicted Drosha/Dicer cut sites. (**c**) Predicted binding of processed shRNA (now siRNA) and cleavage sites of endogenous mRNA (here PTEN)

6. The resultant colonies from the ligation transformation are generally (>99 %) correct, therefore, typically picking one colony to miniprep will suffice. It is however advised to pick at least two colonies in case the quality or concentration of one of the preps is not sufficient for use in the luciferase assays. The concentration of the pBEG shTest DNA is standardized before using it for transfections in the luciferase assays. Thus, clean DNA that is quantifiable is required. This is why use of a miniprep kit is recommended for the isolation of pBEG shTest DNA. Use a miniprep protocol for low-copy plasmids DNA isolation since the DNA yield for shRNA plasmids is usually low.

7. 293T cells are highly recommended although other efficiently transfectable mammalian cell lines can be used.
8. Standardizing the concentration of the DNA to be transfected is highly recommended to reduce variability that could be introduced by using variable transfection volumes.
9. The transfected ratio of pCheck2 to pBEG shTest plasmid DNA is 1:4. Amount of DNA to transfect is calculated according to plasmid size (i.e., bp). Take this into consideration if using another type of shRNA expression plasmid. Transfect the cells with up to 0.74 μg of DNA. Using more DNA can cause toxicity [39].
10. Prepare a master mix of Opti-MEM and PEI to dispense into the microtubes containing the pBEG shTest and pCheck2 DNA. Alternative transfection reagents can be used but we have found that PEI works very well and is the most cost-effective for this purpose.
11. The luciferase assay solutions (LAR II and Stop & Glo® Reagent) can be made in the lab instead of buying the Promega Dual-Luciferase Assay kit. These can be prepared as described previously [40, 41]. Cells should be lysed using the Promega lysis procedure with Promega Passive Lysis Buffer, which is available to purchase separately from the kit.
12. Before lysis, the 293T cells can be checked microscopically for expression of eCFP from the pBEG shTest plasmid to ensure the cells were transfected efficiently. The best results are obtained when ≥30% of the cells are expressing eCFP.
13. The most efficient method to collect the luciferase assay measurements from a large number of samples is by using a luminometer configured to read multiple sample tubes or a 96-well plate in a plate-reader format. Furthermore, sample processing is much faster if the luminometer is equipped with two reagent injectors. If this is the case, the desired volume of sample lysate is first dispensed into the sample tubes or 96-well plate followed by sequential injection of LAR II and Stop & Glo® Reagent by the luminometer instrument. If using an automated program for the injection, shaking, and measurement steps, program the instrument to inject 100 μL of LAR II, shake for 2 s, acquire the Firefly luminescence reading over 10 s, inject 100 μL of Stop & Glo® Reagent, shake for 2 s, and acquire the Renilla luminescence reading over 10 s, in that order.
14. Up to 20 μL of lysate can be used for luminescence measurement. For high efficiency transfections (293T), do not use more than 10 μL of lysate since readings are beyond the linear range of detection for the luminometer. It is important to verify the luminometer is set to display a diagnostic error when

the luminescence exceeds the linear range of the detector. For low efficiency transfections (other cell lines), up to 20 µL of lysate can be used.

15. See pBEG entry plasmids and pLEG destination plasmids our lab has deposited at http://www.addgene.org/browse/article/7497/ (constructed as described in ref. [36]). Available pBEG R2-L3 plasmids contain a drug selection cassette (puromycin, neomycin, hygromycin, and blasticidin) or a fluorophore cassette (eGFP, eCFP, or dsRed) (Fig. 2).
16. Depending on the strain of bacteria used, white/clear screening of transformed bacterial colonies can help with selecting colonies containing the correct recombinant pLEG plasmid [42]. For example, if transforming DH10B bacteria with the recombination reaction, clear colonies will nearly always (>99%) contain the desired recombinant plasmid, whereas opaque colonies will never contain the correct construct. The colonies must be checked 13–16 h after plating otherwise they will all begin to appear white as they grow denser. This method of screening permits the medium/high-throughput production recombinant lentiviral vectors.
17. It is recommended to seed an extra plate of cells that will not be transduced but will be cultured in the selection drug during the selection period. This will serve as a control that can be monitored to determine when all the non-transduced cells have died from incubation in the selection drug.

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