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An advanced blue–white screening method for construction of shRNA expression vectors

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ABSTRACT

Short hairpin RNA (shRNA) encoded within an expression vector is an effective tool for exploration of gene function in mammalian cells. Many of the current methods for constructing shRNA expression vectors require cumbersome and time-consuming procedures for identification of the desired recombinants. We have developed a highly efficient and less labor-intensive cloning method that allows the construction of shRNA expression vectors in one day and with minimal effort. This advanced blue–white screening technique was developed by combining the reconstitution of ideal *lacO* with TA cloning. The DNAs are simply ligated into the destination vectors and, following transformation, a desired recombinant event will give a typical blue colony. In addition, we have used this cloning method for the construction of targeting reporter expression vectors to measure the efficacy of the corresponding shRNA. We constructed 122 functional shRNA expression vectors and sequencing of the positive cloning vectors confirmed a high degree of accuracy. Only three short DNA primers are needed for constructing both shRNA and targeting reporter expression vectors. This advanced blue–white screening system is a powerful tool for the high-throughput assay of RNAi libraries.

Introduction

RNA interference (RNAi) is a sequence-specific, post-transcriptional, gene silencing process. Rapid gene silencing by RNAi is a powerful tool for studying the function of genes on a genome-scale in cultured mammalian cells. Gene silencing can be induced by direct transfection of cells with chemically synthesized [1] or in vitro transcribed siRNA [2]. Alternatively, it can be obtained by transfecting a plasmid or transducing a viral vector encoding a short hairpin RNA (shRNA) driven by an RNA polymerase (pol) III promoter and tRNA promoters [3–5], or a pol II promoter [6–8]. Compared to siRNA, shRNA has the advantage of greater silencing longevity, more delivery options and lower cost [9].

A typical strategy for the construction of shRNA expression vectors in mammalian cells requires synthesis, annealing and ligation of two long, complementary oligonucleotides consisting of the whole shRNA with a terminal signal of 5 or 6 nucleotides and extra nucleotides for cloning [10]. This strategy is useful for the construction of shRNA expression vectors; however, it suffers from a high rate of mutation and the high cost of producing long oligonucleotides. Many other methods eradicate or greatly minimize the mutation problem, but most of them require at least one long oligonucleotide, which is costly or needs special processing [11–14]. Furthermore, many cloning methods used for the construction of RNAi expression vectors, including those mentioned above, cannot ensure that all the transformants are 100% recombinants. Most of these methods include a series of procedures for screening the desired recombinants, such as DNA isolation, PCR amplification, enzyme digestion, and electrophoresis. There is a need for a more versatile and more cost-effective method for the rapid generation of shRNA expression vectors.

We have developed an advanced blue–white screening method for the generation of both shRNAs and targeting reporter expression vectors. This simple and inexpensive approach is efficient, reliable, and independent of purification, restriction enzyme digestion of target DNAs and the synthesis of long primers. Only one universal and two specific synthetic short DNA primers are required for construction of both the shRNA and corresponding targeting reporter expression vectors. In this study, 122 shRNA expression vectors were constructed with a cloning efficiency of 98% and the mutation rate of positive shRNA clones was reduced to 15%. In addition, by using fully analysed shRNA vectors directly against expression of exogenous genes we have demonstrated that this shRNA cloning system is both effective and faithful. This new method is suitable for the high-throughput assay of RNAi libraries (Fig. 1).

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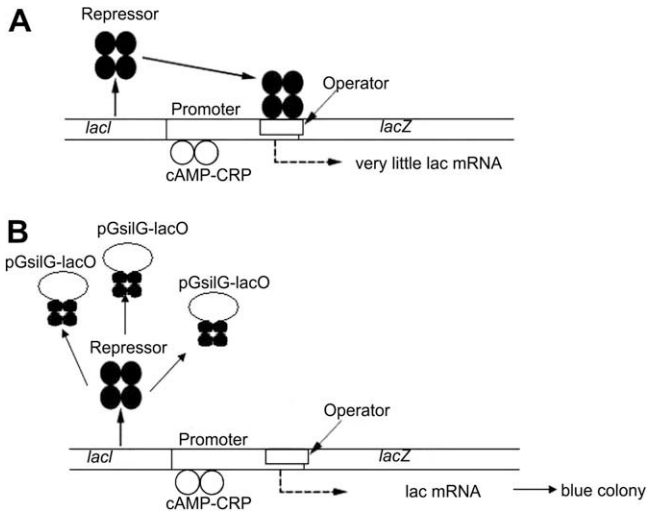

Fig. 1. The mechanism of operator–repressor titration in *Escherichia coli*. (A) Normally, when no inducer or plasmid with *lacO* is introduced, the endogenous lacZ promoter is repressed by lac repressor bound to its *lacO* sites. (B) When a high-copy number plasmid possessing *lacO* sites is introduced into the cell, the lac repressor molecules are bound by plasmids, thereby derepressing transcription of the *lacZ* promoter, giving a blue colony.

Materials and methods

Construction of shRNA TA cloning vector pGSilG-lacOΔ and targeting reporter TA cloning vector psiCHECK-2GlacOΔ. Each of the BfuI-recognizing sites (5′-gtatcc-3′) in vector pGenesil1.0 (Genesil Biotechnology, Wuhan, China) and psiCHECK-2 (Promega) was replaced by a BamHI site (5′-ggatcc-3′) using a site-directed mutagenesis strategy [15]. The TA cloning vectors pGSilG-*lacO*Δ (Fig. 2A) and psiCHECK-2G*lacO*Δ (Fig. 2B) were constructed by inserting the GFP gene from pGsilG [8] into pGenesil1.0 and psiCHECK-2, respectively. The GFP cassette was flanked by two BfuI restriction sites, and a 13/20 bp ideal *lacO*Δ sequence was added to the 3′end of the *gfp* expression cassette (5′-agc gct cac aat t-3′). The primers used for construction of the TA cloning vectors are given in Table 1. Prime STAR HS High-Fidelity DNA Polymerase (TaKaRa, Dalian, China) was used for all of the PCR reactions for plasmid construction.

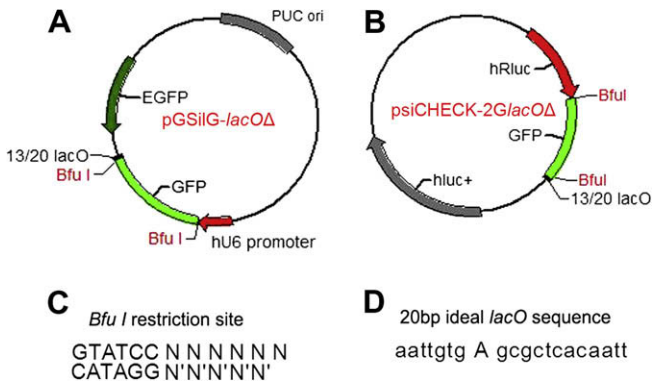

Fig. 2. ShRNA TA cloning vector and targeting reporter TA cloning vector. (A) A map of the pGSilG-*lacOΔ* vector. A GFP cassette is inserted after the hU6 promoter. The GFP cassette can be expressed only in *E. coli*. EGFP can express green fluorescence in HEK293T or HeLa cells but not in *E. coli*, so it does not conflict with the GFP cassette. EGFP expression provides a visual indication of the transfection efficiency. (B) A map of the psiCHECK-2G*lacOΔ* vector. The Renilla luciferase cassette, hRluc, is used to monitor changes in expression as the result of RNAi induction. The firefly reporter cassette, hluc+, is an intraplasmid transfection normalization reporter. Thus, when using the psiCHECK-2G*lacOΔ* vector, the Renilla luciferase signal can be normalized to the firefly luciferase signal.

Table 1
The primers used for construction of shRNA TA cloning vector pGSilG-*lacO*Δ and targeting reporter TA cloning vector psiCHECK-2G*lacO*Δ.

Primer name	Primer sequence (5′–3′)	T_m (°C)
1F	ggatccctgtccgcctttctccc	58
1R	cctgccgagaaagtgtccatcatgg	58
2F	ggacactttctcggcaggagcaagg	57
2R	ggatccgctcatgagacaataaccctg	57
3F	gttattgtctcatgagcggatccatatttgaatg	60
3R	aaaggcggacagggatccggtaagc	60
4F	ggatccgctcatgagacaataaccc	58
4R	ggatccctgtccgcctttctcc	58
5F	ggagaaaggcggacagggatcc	58
5R	gggttattgtctcatgagcggatcc	58
6F	ttcttcggatacggatccctaagcccaatgtgtttttttc	60
6R	aattgtgagcgctctagaggatacttcacttgtacagctcgtccatgcc	57
7F	tctagagcgctcacaattaagcttcccgggacgcgttaag	59
7R	agggatccgtatccgaagaaggatcccgcgtcctttccac	57
8F	ctcgagttcttcggatacggatccctaagcccaatgtgtttttttc	59
8R	gcggccgcaattgtgagcgctctagaggatacttcacttgtacagctcgtccatgcc	59

Primers 1F, 1R, 2F, 2R, 3F, and 3R were used for pGenesil1.0 mutation, and 4F, 4R, 5F, and 5R were used for psiCHECK-2 mutation. Primers 6F and 6R were used for the amplified GFP cassette to construct pGSilG-*lacO*Δ. Primers 7F and 7R were used for the amplified linearized mutated pGenesil1.0 to construct pGSilG-*lacO*Δ. Primers 8F and 8R were used for the amplified GFP cassette to construct psiCHECK-2G*lacO*Δ. Mutation sites are in bold letters, the 18–20 nt overlapping sequence for overlap PCR is singly underlined, and the self-circling homologous sequence is doubly underlined.

Construction of shRNA expression vectors and targeting reporter expression vectors by an advanced blue–white screening method. The following steps were used to construct a human U6 promoter-driven shRNA vector with a recommended loop sequence of 5′-TTCAAGAGA-3′ as proof of principle. The entire procedure involves the following three steps, as shown in Fig. 3A.

Step 1: Primer design. Two short specific primers were designed for blue–white screening. The sequence of the forward primer was 5′-$N_{(19-23)}$ ***TTC AAG AGA***-3′ and that of the reverse primer was 5′-CAC AAT TAA AAA A$N_{(19-23)}$ ***TCT CTT GAA***-3′. The 5′ loop sequences are necessary for primer extension. For instance, if the target sequence is 5′-CCA CAC AAC CTG GTA GCA T-3′, the shRNA structure should be 5′-CCA CAC AAC CTG GTA GCA T***TT CAA GAG A***AT GCT ACC AGG TTG TGT GGT TTT TT-3′. Thus, the sequence of the forward primer should be 5′-CCA CAC AAC CTG GTA GCA T***TT CAA GAG A***-3′ and that of the reverse primer should be 5′-CAC AAT T AA AAA ACC ACA CAA CCT GGT AGC AT***T GTC TTG AA***-3′. All primers were purchased as general standards, purified by PAGE (Invitrogen) and suspended in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA) at a concentration of 10 pM/μL. The short length of the primers (⩽43 nt) ensures their quality.

Step 2: Primer extension process. A 25 pmol sample of each oligonucleotide was used in the extension reaction, which included LA Taq PCR buffer, 0.2 mM (final concentration) each dNTP, and 1 U of LA Taq polymerase (TaKaRa, Dalian, China). The reaction was done at 30 °C for 20 min and then at 72 °C for 10 min (Fig. 3A and B).

Step 3: Ligation and transformation. A 1.3 μL (approximately 100 ng) sample of the primer extension fragments (without purification) were ligated directly into 0.2 μL (about 30 ng) of linearized T vector (pGSilG-*lacO*Δ) in solution I (TaKaRa, Dalian, China). The linearized T vector was prepared by digestion of pGSilG-*lacO*Δ with BfuI (BioLabs, England). The ligation mixtures were transformed into 50 μL portions of DH10β [16] chemically competent cells. Transformants were selected by spreading 200 μL of transformation liquid on an LB plate supplemented with kanamycin (25 μg/ml) and X-gal (30 μg/ml) and incubated at 37 °C for 12 h in a light-shading incubator. The blue transformants on the plates re-

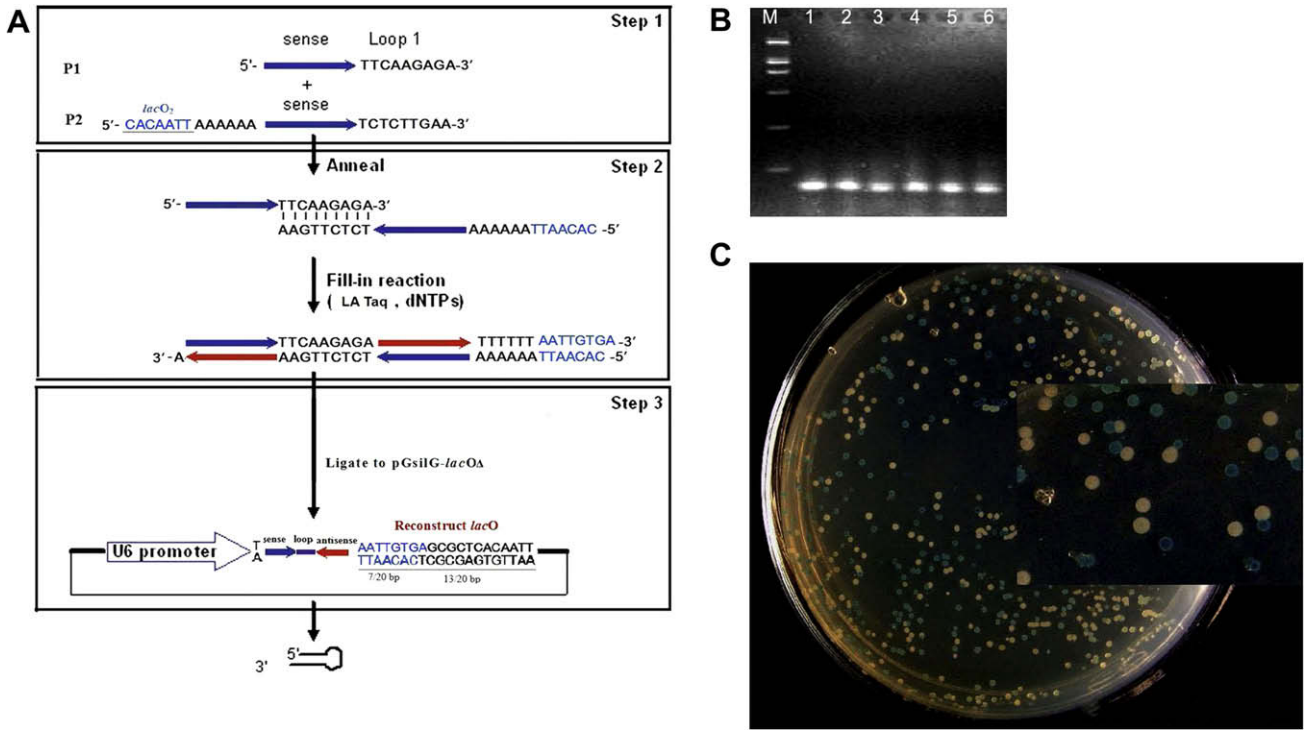

Fig. 3. Main procedures for the visible identification cloning strategy to generate shRNA expression vectors. (A) An overview of the primer extension method used to produce short hairpinDNA inserts for construction of the shRNA vector. (B) Analysis of 2 μL of primer extension products on a 3% agarose gel. (C) Following routine ligation and transformation, the desired recombinants are blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

vealed the desired recombinants. Positive clones were selected and confirmed by automated sequencing using the hU6 sequencing primer (5′-CCA AGG TCG GGC AGG AAG AG-3′).

Corresponding targeting reporter expression vectors were constructed by the same steps, except the reverse primer was replaced by a universal primer and the extension product was ligated to linearized T vector psiCHECK-2G*lacO*Δ. The sequence of the universal primer is 5′-CAC AAT T***TC TCT TGA A***-3′. Positive clones were selected and confirmed by automated sequencing using the hRluc sequencing primer 5′-gtgaaggtgaagggcctccacttc-3′.

Cell culture and transfection. Human embryonic kidney (HEK) 293 cells were used to test the efficacy of RNA interference. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (GiBco) at 37 °C in a humidified incubator with a 5% (v/v) CO_2 atmosphere. The cells were transiently cotransfected either with 0.5 μg of the gene expression vector of interest or the targeting reporter plasmid and 1.5 μg of the corresponding shRNA expression plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. At 48 h after transfection, RT-PCR, Western blot and the Renilla luciferase assay were used to determine the efficacy of silencing of the target gene.

RT-PCR analysis. RT-PCR was used to semi-quantify the mRNA expression level of genes. Briefly, total RNA was isolated from cells using TRIzol® reagent (Invitrogen). Reverse transcription reactions were done with Superscript III reverse transcriptase (Invitrogen) and an oligo(dT) primer by following the manufacturer's instructions. A fragment of human endogenous β-actin was amplified simultaneously in each PCR reaction as an internal standard. The PCR products were resolved by electrophoresis in 1.5% (w/v) agarose gel and the bands were visualized by staining with ethidium bromide. The mRNAs were amplified with the primers given in Table 2.

Western blot analysis. Total proteins were extracted from cells using lysis buffer (250 mM sucrose, 1% (v/v) Triton X-100, 2 mM EDTA, 10 mM EGTA, 50 mM Tris–HCl, pH 7.4, 200 μg/mL of leupeptin). Equal amounts of total protein extracts (10–20 μg/lane) from the cell lysates were separated by SDS–PAGE (12% polyacrylamide gel) and transferred electrophoretically onto a nitrocellulose membrane (Immobion™ transfer membrane, Millipore, USA). After blocking non-specific binding sites with 5% (v/v) non-fat milk, the membrane was incubated with primary antibodies for 1 h at room temperature. The primary antibodies used were anti-Flag (Sigma, 1:2000 dilution), and monoclonal anti-β-actin antibody (Sigma, 1:2000 dilution). After washing, the blot was incubated with goat anti-mouse IgG (H + L)-HRP, and immunoreactive bands were visualized using a Super signal maximum sensitivity kit (Thermo Scientific).

Renilla luciferase assay. The luciferase-expressed cells were harvested at 48 h post-transfection and samples of the cell lysates containing equal amounts of protein were measured by the Dual-luciferase Reporter Assay System (Promega, Madison, WI, USA).

Table 2
The RNAi targets used in this paper.

Name	Target
shMyD88	5′ AACTGGAACAGACAAACTATC 3′
shSIAH1	5′ GATCCATTCGCAACTTGGC 3′
shP53	5′ GACTCCAGTGGTAATCTAC 3′
shEGFP	5′ GGCGATGCCACCTACGGCAAG 3′

Results

Construction and confirmation of TA cloning vectors pGSilG-lacOΔ and psiCHECK-2GlacOΔ

Both pGSilG-*lacO*Δ (Fig. 2A) and psiCHECK-2G*lacO*Δ (Fig. 2B) contain a *gfp* expression cassette as a counter-selectable marker to eliminate the background of poorly digested vector. The cas-

sette is flanked by two restriction sites for BfuI. At the end of the GFP cassette, a 13/20 ideal *lacO* sequence was set beside one of the BfuI sites. The shRNA inserts were cloned into the BfuI sites of pGSilG-*lacO*Δ and driven by the hU6 promoter. Short DNA fragments containing an RNAi-targeting sequence were cloned into the BfuI sites of psiCHECK-2G*lacO*Δ and located 3′ to the synthetic hRluc gene. It has been reported that a 19 nt sequence fused at the 3′ UTR of the targeting reporter gene is sufficient for the siRNA validation assay [17]. The shRNA and targeting reporter vectors were designed to have the same clone site to ensure the greatest value and utility of the specific primers of targeters. So, only two specific and one universal short DNA primers are needed for construction of both the shRNA and the targeting reporter expression vectors.

These two vectors were used as negative controls for screening shRNA expression vectors in *Escherichia coli* and for cell transfection. We have confirmed the identity of all the vectors generated as described above by PCR identification, enzyme digestion and sequencing.

Strategy and experimental design for the advanced blue–white screening method

We have devised a genetic method for identifying the desired directional recombinants automatically in order to screen and identify the desired recombinants more effectively and conveniently. In detail (see Fig. 3), we used a 20 bp *lacO* act as a selectable marker [16,18]. The extended short DNA insert was designed with a tail of 5′ 7/20 ideal *lacO* sequence (5′-aattgtgA-3′, up strand) downstream, and the T vector was designed to have the 3′ 13/20 ideal *lacO* sequence (3′-Tcgcgagtgttaa-5′, lower strand) on the 3′ end of the vector (Fig. 3A). Following routine ligation and transformation, insertion of a target sequence in the correct orientation resulted in the construction of the ideal *lacO* (5′-aattgtg A gcgctcacaatt-3′, up strand). When *lacO* sites are present on the recombinant plasmid, the *lac* repressor molecules are bound by the high-copy number recombinant plasmid, thereby inducing the endogenous lac promoter driving the β-lactamase gene, resulting in a typical blue colony (Fig. 3C). The blue transformants revealed

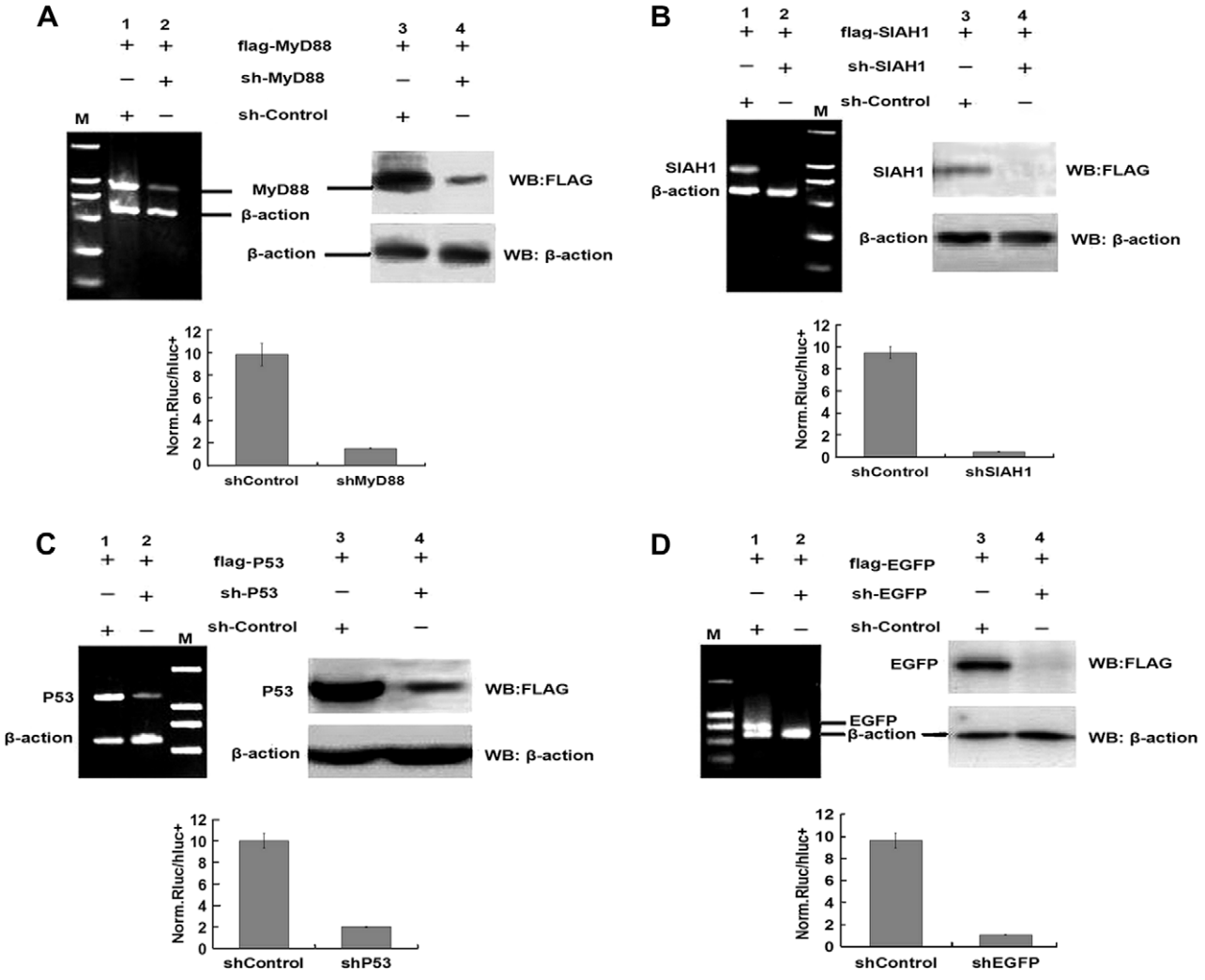

Fig. 4. RT-PCR and Western blot showing specific suppression of MyD88, SIAH1, p53, and EGFP in transfected HEK293 cells. (A) HEK293T cells were cotransfected with a vector encoding flag-MyD88 and a MyD88-specific shRNA vector (indicated by shMyD88) or a negative control vector (shControl). After incubation for 48 h, total RNAs were prepared and subjected to reverse transcription (RT)-PCR for MYD88 and β-actin mRNAs (*left-hand panel*). Cell lysates were subjected to Western blot with anti-FLAG antibody (*right-hand panel*). HEK293T cells were cotransfected with a targeting reporter vector of MyD88 and a MyD88-specific shRNA vector (indicated by shMyD88) or a negative control vector (shControl). After incubation for 48 h, cell lysates were subjected to luciferase assay. The Renilla luciferase/firefly luciferase (hRluc/hluc+) ratio was normalized and calculated against the control vector. The data plotted are averaged from three independent experiments and the bars indicate the standard deviation (*lower panel*). (B) The RT-PCR (*left-hand panel*), Western blot (*right-hand panel*) and luciferase assay (*lower panel*) results for SIAH1 gene silencing. (C) The RT-PCR (*left-hand panel*), Western blot (*right-hand panel*) and luciferase assay (*lower panel*) results for p53 gene silencing. (D) The RT-PCR (*left-hand panel*), Western blot (*right-hand panel*) and luciferase assay (*lower panel*) results for EGFP gene silencing. The methods of analysis are described above.

Table 3
The primers used in RT-PCR.

MyD88-F	5′ ATGCGACCCGACCGCG 3′
MyD88-R	5′ GGGCAGGGACAAGGCCTTG 3′
SIAH1-F	5′ ATGAGCCGTCAGACTGCTACAGC 3′
SIAH1-R	5′ ACACATGGAAATAGTTACATTGATGCCT 3′
P53-F	5′ ATGGAGGAGCCGCAGTCAGA 3′
P53-R	5′ GTCTGAGTCAGGCCCTTCTGTCTT 3′
EGFP-F	5′ ATGGTGAGCAAGGGCGAGGA 3′
EGFP-R	5′ TTACTTGTACAGCTCGTCCATGCCG 3′
β-Actin-F	5′ GGCATCGTGATGGACTCCG 3′
β-Actin-R	5′ GCTGGAAGGTGGACAGCGA 3′

the directional recombinants. To check the recombinants and the orientation of the insertion, blue colonies were picked and cultivated for plasmid isolation, and the desired recombinants were confirmed by sequencing.

Next, we needed a method to achieve TA cloning. In this study, type II restriction enzyme BfuI was introduced into the destination vectors at the desired point. The BfuI enzyme recognized the fixed sequence 5′-gtatcc-3′ and digested the target DNA at 6 bp downstream with a random sequence, which left a single nucleoside (T) on the 3′ end of the vector (Fig. 2C). This method also makes a seamless clone available for TA cloning. The entire scheme for visible identification of the desired recombinant clones is illustrated in Fig. 3.

Sequencing of positive clones

One or two blue clones were selected randomly for automated sequencing, and the results were satisfactory: nearly all blue colonies possessed directional recombinant DNA molecules. Only 18 out of 122 (14.7%) shRNA expression plasmids contained a mutation. These results indicated that the new method is less error-prone than the commonly used approaches, which have a mutation rate of 25–50%. Only 1 out of 30 (3.33%) targeting reporter expression vectors contained a mutation.

Transient gene silencing efficacy with shRNA expression vectors

We present some representative RT-PCR, Western blot and Renilla luciferase assay results for MyD88 (myeloid differentiation primary response gene 88; NCBI-GeneID 4615), SIAH1 (seven in absentia homolog 1 (*Drosophila*); NCBI-GeneID 6477), p53 (*Homo sapiens* tumor protein p53; NCBI-GeneID 85417) EGFP (enhanced green fluorescence protein) RNAi in Fig. 4. The targets selected and the primers used in RT-PCR are given in Table 2 and Table 3, respectively.

Discussion

We have developed an advanced blue–white screening method for the generation of shRNA and targeting reporter expression vector. This simple and inexpensive approach is efficient, reliable, and independent of purification, restriction enzyme digesting target DNAs and the synthesis of long primers. The method is less labor-intensive than those commonly used at present, and the high level of cloning efficiency is achieved by the reconstruction of a *lac* operator sequence as a selectable element. Meanwhile, the low mutation rate is ensured by using a primer extension method with short primers.

A common drawback of all current methods for constructing shRNA vectors is the use of tedious and difficult processes to identify the recombinants, such as DNA isolation, PCR amplification, enzyme digestion, and electrophoresis. However, true positive-selection methods have not been employed for the construction of shRNA expression vectors because the inserts are extremely short. The traditional positive-selection method involves either the inactivation of a genetic marker or the replacement of that marker by the target gene [19–22]. So, a short DNA insertion into the reporter gene's frame might make the reporter gene's product work. In this study, instead of destroying the reporter gene's function, we designed the vectors and the inserts to reconstitute the ideal *lacO*; thus, the reconstituted ideal *lacO* on the high-copy number vector competed the repressor LacI with the *lacO* on the promoter of Plac, which derepressed the transcription of *lac*Z and turned the colonies blue. All of the screening work is very simple; the researcher needs only to glance at the plates and sieve out the blue colonies for sequencing.

The high level of cloning efficiency of this method is achieved by using a reconstructed *lac* operator sequence as a selectable element. First, a badly prepared T vector can be picked out because the colonies produced by a badly digested plasmid will not be blue because of the defective *lacO*. Second, all the T vectors are designed to contain the expression cassette of *gfp* flanked by two BfuI sites. Colonies produced by undigested vectors will give green fluorescence, and will not turn blue. Third, the transformants containing inverted insertions and self-circled vectors cannot become blue because only the recombinant DNA molecules with 7/20 ideal *lacO*-tailed fragment inserted correctly can coactivate endogenous *lac*Z gene transcription and turn the host colony blue. Colonies with a typically blue color revealed the desired recombinants.

Typically, 25–50% of cloned shRNA constructs contain significant mutations as determined by DNA sequencing. The mutation frequency is close to 75% when the desired siRNA sequence is 29 nt long [11]. The unreliability is due, in part, to errors in oligonucleotides >50 nt. The two complementary oligonucleotides encoding a desired shRNA target sequence, which are almost 60 nt long, are difficult to synthesize and are unreliable. In this new approach, however, the accuracy of positive clones is improved dramatically by the primer extension strategy. Two shorter primers ($\leqslant$43 nt) are suitable for the primer extension strategy and can reduce the possibility of mutations dramatically. The shortest forward primer is only 28 nt and the shortest reverse primer is only 40 nt. Primer quality is associated with the degree of sequence accuracy and all primers should be purified by PAGE before use. Because our protocol is based on conventional TA cloning, the extension products can be ligated directly into the destination vectors without the need for enzyme digestion.

In this study, all of the plasmids were sequenced and we found that only 18 of the 122 plasmids (14.7%) had a mutation, which was nearly always a sequence deletion in the 5′ end of the target. The deletion mutations might be introduced during primer synthesis in the 3′–5′ direction, so the primers were mixed with many incomplete synthesised sequences. However, extension and ligation reactions can still be done with the incompletely synthesised sequences. The reconstruction of *lacO* allows the selection of positive recombinants on agar plates, and ensures the integrity of the reverse primer 5′ end; so, fewer mutations occurred when the quality of the primers was assured.

In summary, we have developed an advanced blue–white screening method for construction of both shRNA and targeting reporter expression vectors with a requirement for only two specific and one universal synthetic short DNA primers. Because of its simplicity and effectiveness, this approach may be useful for large-scale analysis of mammalian gene function.

Acknowledgments

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