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Fluorescence based evaluation of shRNA efficacy

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Abstract

RNA interference is a cellular mechanism regulating levels of mRNAs. It has been widely exploited to knockdown specific protein targets. The selected interfering RNA sequence greatly influences its ability to knockdown the target. Here we present a method for constructing multiple testing plasmids which express small hairpin RNAs (shRNA) targeting different regions of an mRNA. A simple fluorescence test in cultured cells allows convenient evaluation of mRNA knockdown by many different shRNAs on 96-well plates. We show that software predicted shRNAs have varying efficacies and only 2 of the 7 tested shRNAs significantly knocked down their targets.

Keywords

shRNA; interfering RNA; knock down; gene silencing; in vitro screening

RNA interference is a biological process which allows for the controlled degradation of specific mRNA targets. The mRNA target is specified by the sequence of the small-interfering RNA (siRNA). Small hairpin RNA (shRNA) is similar to siRNA, but can be expressed continuously in a plasmid or a viral vector. Bioinformatic studies have produced rules for selecting the sequences of siRNA. Computer programs that utilize these rules are available for predicting the sequences that are likely to be effective. However, some of the predicted sequences are much more effective than others and validation is necessary. Frequently used methods to confirm effective RNA knockdown include qPCR, flow cytometry, western blot or immunohistochemical techniques, which can be tedious and time consuming [1; 2; 3]. More convenient, rapid methods of evaluating shRNA efficacy would greatly expedite interfering RNA research. Kamio et al [1] recently described a method using GFP as a reporter to evaluate target gene knock down. Here we report a different design of the similar approach, also using GFP as a reporter, with several new features that make the shRNA evaluation more convenient, rapid, and affordable.

The design of shRNA usually starts with using computer programs to predict the sequence of siRNA that will likely be able to knockdown a specific target. There are also a number of commercial sources available where shRNAs can be purchased. However, novel shRNAs

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from these sources are made based on software predictions and have not been validated. We have developed a system for easily making a large number of shRNA constructs and screening for those with the highest efficiencies of knocking down targets expressed in cultured cells. First, a cDNA fragment of a target mRNA is fused with green fluorescence protein (GFP) cDNA to form a single transcript. Thus the intensity of green fluorescence expressed by this reporter plasmid allows tracking of the target mRNA knockdown. Second, shRNA plasmids are constructed by inserting annealed oligonucleotides with desired sequences into a restriction site of our shRNA expressing vector. Within these plasmids, the shRNA sequences are fused with a red fluorescence protein (RFP) cDNA, controlled by an inducible promoter. Therefore, the intensities of the red fluorescence represent the expression level of the shRNA. The target plasmid and a shRNA plasmid are cotransfected into cultured cells in 2 sets of wells of a dish and doxycycline is added to one of the 2 sets to induce RFP-shRNA expression. Cells in the uninduced wells will exhibit strong green fluorescence but no red fluorescence, while cells in the induced wells will show strong red fluorescence and reduced green fluorescence as compared to the uninduced control wells if the shRNA is efficacious. Once the degradation complex recognizes the target sequence specified by the shRNA, the entire transcript is degraded, including the GFP portion of the mRNA [4]. Therefore, the extent of green fluorescence reduction indicates the effectiveness of the shRNA at knocking down its mRNA target. Many different shRNA plasmids can be constructed in the same ligation procedure and many shRNA can be easily evaluated in a single 96-well plate.

Our GFP reporter vector contains a CMV promoter followed by a nonstable GFP (GFPns) cDNA and a BGH polyA signal. The GFPns form of GFP has a shorter half life and thus reflects the mRNA knockdown more readily [5]. The GFP reporter construct is made by inserting a piece of DNA representing the entire targeted mRNA or a fragment of it after the stop codon of the GFPns and before the polyA signal (Figure 1A). The plasmid will express a single transcript with GFPns being translated into protein and the targeted mRNA acting as the 3' untranslated region (3'UTR). If the shRNA being tested is capable of knocking down its target mRNA, its co-expression will lead to the degradation of the entire transcript including the GFPns coding region. This results in a significant reduction in GFP expression. Moreover, since this fluorescence reduction can be quantitatively measured with ease, efficacies of different shRNA sequences at knocking down the same mRNA target can be quantitatively compared.

Our shRNA expressing construct was derived from pTRIPZ shRNAmir vectors (Open Biosystems, Huntsville, AL)[2; 6]. Figure 1B shows the major components of the plasmid. A tetracycline-inducible promoter (Ptet) was used to drive the expression of turboRFP (tRFP) [7] with a shRNA structure as its 3'UTR. The expression level of tRFP allows one to track the expression levels of the shRNA. The shRNA structure contains the 5' and 3' flanking sequences (5'mir, 3'mir) and the loop sequences of microRNA-30 because these sequences have been shown to significantly increase the Drosha and Dicer processing of the expressed hairpins and thus the effect of RNA interference [3]. The double stranded stem portion of the shRNA defines target specificity. We have made a shRNA vector by inserting a 1.3kb filler DNA fragment flanked by two BspQ1 restriction enzyme sites between the 5'mir and 3'mir sequences. Once digested with BspQ1, the double digested vector then has two incompatible 5' overhang ends and can be easily isolated from any single digested vector, thus eliminating self-ligation background in later procedures. The digested vector is ready for the insertion of the shRNA sequence, which is generated by annealing two single stranded oligonucleotides to form a small double stranded insert with proper overhangs as shown below.

5'–
 gcgCTGGGAGTTTCCCAGTGAACAGTAGTGAAGCCACAGATGTACTGTTTCAC
TGGGAAACTCCCAT –3'

3'–
GACCCTCAAAGGGTCACTTGTCATCACTTCGGTGTCTACATGACAAGTGAC
CCTTTGAGGGTAacg –5'

This short insert contains the target specifying sequences (underlined, which can be replaced with other siRNA sequences) and the microRNA-30 loop (in italics; which would remain the same regardless of the siRNA sequence used). This is a very efficient and cost effective method for generating many different shRNA expressing constructs in a single procedure. Our shRNA vector also contains the 5' and 3' long terminal repeats (LTR) of the adenyl associated virus (AAV) so that they could be packaged into virus particles without the need for further subcloning.

We applied this methodology to evaluate the shRNA constructs targeting specific isoforms of the D2 dopamine receptor, which is described as an example of this method in practice. The D2 dopamine receptor has two different splice variants, a short isoform (D2S) and a long isoform (D2L) which contains 29 additional amino acids from exon 6. Our goal was to design a vector that knocked down only the D2L splice variant (shRNA D2L) while leaving D2S mRNA expression unaffected. We used a computer program, siRNA wizard (v3.1, in vivogen), to predict effective RNAi target sequences and chose 7 sequences for validation. Oligonucleotide pairs were annealed and inserted into our shRNA expressing vector. We then transfected Chinese Hamster Ovary (CHO) cells with both the reporter and shRNA expressing constructs in 96 well plates using lipofectamine (Invitrogen, Carlsbad, Ca). Doxycycline was added to half of the transfected cells 3 hours after transfection and examined 2–4 days later with a fluorescence microscope. Figure 2 shows the GFP expression of the reporter construct (in green) as well as RFP expression from the shRNA construct (in red). As can be seen in Figure 2, RFP expression is only visible in wells with doxycycline induction (lower panels), and GFP expression is substantially lower as the D2L specific shRNA degrades the GFP-D2L fusion mRNA transcript, compared to those wells without induction (Figure 2D versus 2A). This effect was isoform specific, as GFP expression from a GFP-D2S construct was not affected by the same shRNA construct (data not shown). Figure 2F is the merged image of GFP and RFP images, showing that most RFP expressing cells had no or low GFP expression, indicating efficient D2L knockdown. Also shown in Figure 2F, a few cells expressed both GFP and RFP (yellow cells) indicating insufficient knockdown in these cells, which could be due to more reporter plasmid than the shRNA plasmid being transfected into these cells. The results are consistent with the fact that shRNA knockdown usually does not reach 100%. However, the difference between an effective shRNA and an ineffective one is readily observable in our fluorescence assays in multiple wells for each condition. The fluorescence intensities can be quantitated using the software ImageJ (free public domain software, National Institute of Health, MD). As shown in figure 2G, the 7 shRNAs made according to software predictions had different efficacies in knocking down D2L mRNA and only 2 out of the 7 shRNAs knocked down their target significantly (ANOVA with a Tukey Pot-hoc analysis. *p<0.05. SPSS 18.0, IBM, NY). This result strongly suggests that validating shRNA is necessary. If one were to simply pick a computer generated RNAi sequence without validation, the chance of getting an effective knockdown is less than 30%.

There are several differences between our method and the method reported by Kamio et al [2]. To avoid variations due to different transfection conditions, we used inducible shRNA expression, and thus transfection conditions can be kept constant. We demonstrated that a fluorescence microscope and the free software ImageJ can be used to acquire and analyze

GFP expression data. In addition, we have introduced an inexpensive and high throughput way of constructing many shRNA constructs in a single cloning step. We (and Kamio et al) have shown that certain computer predicted sequences are more effective than others in triggering the RNA interference mechanism in cultured cells. While *in vitro* efficacy of a shRNA does not necessitate *in vivo* efficacy, we believe that this screening test should greatly increase the chance of success for *in vivo* studies.

We present here a simple method for evaluating multiple shRNA sequences in knocking down their mRNA targets in cultured cells using fluorescence as indicators. It is relatively rapid and easy compared to histochemical or qPCR confirmation. We also show an inexpensive way of making many different shRNA constructs by annealing pairs of oligonucleotides and ligating directly into our shRNA vector. Finally, the ability to induce shRNA expression in a portion of identically transfected cells allows more controlled comparison of the cells with and without knockdown. Taken together, we describe here a convenient, inexpensive, rapid and thus high-throughput suitable method for simultaneously evaluating the efficacies of multiple different shRNAs at knocking down specific mRNA targets.

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Figure 1.

Reporter construct and shRNA construct. (A) A CMV promoter drives expression of a single transcript containing the GFPns coding sequence followed by a piece of the target cDNA sequence and a polyA stop signal. The cDNA sequence acts as a 3'UTR and only GFPns protein is synthesized. (B) A tetracycline-inducible promoter (Ptet) drives the expression of the RFP-shRNA transcript with the shRNA acting as the 3'UTR. Different double stranded shRNA sequences with compatible ends, made by annealing pairs of oligonucleotides, can be readily ligated into the two BspQ1 restriction sites between the 5'mir30 and 3'mir30 regions. The Ubiquitin C driven reverse transactivator (rtTA) is also in the plasmid, which is necessary for inducible expression.

Figure 2.

Knockdown of the specific mRNA targets by shRNA expression. CHO cells were cotransfected with a reporter construct expressing GFPns-D2L fusion transcript and tRFP-shRNA constructs targeting D2L. Panels A–C shows the same field of cells in a well without doxycycline induction viewing through a green filter, a red filter, or a merged view of the two. Panels D–F are similar but showing cells in a well with doxycycline induction. The presence of doxycycline induced strong tRFP-shRNA expression (Panel E versus B) paralleled by substantially lower GFP-D2L expression (Panel D versus A). Panel G shows results of quantitation using ImageJ analysis of fluorescence expression. Each bar represents the average GFP expression of 6 wells presented as the ratio of GFP expression to that in the uninduced control wells. Only sequences A and D were able to reduce GFP expression significantly ($p < 0.05$) compared to the control wells.