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One-oligonucleotide method for constructing vectors for RNA interference[1]

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Key words

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Abstract

Aim: To develop an easy, fast, automated, and inexpensive method for constructing short-hairpin-RNA cassettes for RNAi studies. **Methods:** Using single oligonucleotides, a variety of DNA cassettes for RNAi vectors were constructed in only few minutes in an automated manner. The cassettes, targeting the eGFP, were cloned into plasmids driven by RNA polymerase III promoter H1. Then, the plasmids were transfected into HeLa cells that were later infected with a recombinant adenovirus encoding the eGFP gene. The level of eGFP fluorescence was evaluated by confocal imaging and flow cytometry. **Results:** The plasmids constructed with the DNA cassettes made by the one-oligonucleotide method inhibited eGFP with different potencies, ranging from 55% to 75%. **Conclusion:** By using the method reported here, it is possible to simultaneously construct hundreds of different DNA cassettes for RNAi experiments in an inexpensive, automated way. This method will facilitate functional genomics studies on mammalian cells.

Introduction

RNA interference (RNAi) is a powerful tool for inhibiting gene expression in a wide variety of organisms. When double-stranded RNA is introduced into cells, the ribonuclease III Dicer processes the double-stranded RNA into small fragments of about 21 nucleotides in length (termed siRNAs) that trigger the RNAi mechanism[1–3]. Then, the siRNAs are incorporated into a protein complex, known as the RNA-induced silencing complex (RISC), which in turn unwinds the duplex siRNA in an ATP-dependent manner[4]. After the unwinding, RISC uses the siRNA antisense strand as a guide to specifically cleave the complementary mRNA, which is further processed for degradation[4–6].

There are different ways to induce mRNA degradation using RNAi. Several methods for preparing siRNA have been developed, such as chemical synthesis, *in vitro* transcription, siRNA expression vectors, and polymerase chain reaction (PCR) expression cassettes. For example, short hairpin RNAs (shRNAs) transcribed by RNA polymerase III promoter-based vectors have been used as RNAi triggers in a variety of cell lines[7,8]. These RNA polymerase III (RNA Pol) vectors have a variety of advantages over the siRNAs. First, they greatly reduce the cost of RNAi synthesis, making RNAi a viable tool for screening the function of large numbers of genes. Second, because hairpins are transcribed inside cells, it is possible to establish inducible systems for RNAi both *in vitro* and *in vivo*[9–11]. Third, it is also possible to generate knock-down phenotypes to resemble knock-out animals without affecting the germline, as long as this strategy is combined with inducible systems[12–14]. Therefore, it is relatively easy to establish cell cultures using retroviral or adenoviral vectors that stably express the hairpin for RNAi[10,15–18].

Although there has been much progress in the RNAi field, it is not completely understood why some sequences are refractory to RNAi. Some groups have investigated this lack of activity, but at present, choosing the most effective siRNA sequence to knock down an mRNA is still a trial-and-error task. Thus, it is generally accepted that several se-

quences must be designed to achieve the highest RNAi efficiency, making the silencing experiments costly and slow. It would be advantageous to have a more efficient method for the production of a wide variety of sequences in a simple step, instead of using long DNA oligonucleotides or several PCR step-specific methods[8,19–21].

Here we describe an inexpensive, easy to implement and automated method to produce DNA cassettes for RNAi experiments using a single oligonucleotide. With this method hundreds of DNA cassettes can be synthesized in a 1-step reaction. This method not only reduces the cost of testing sequences for RNAi experiments, but also accelerates the exploration of multiple-gene sequences.

Materials and methods

Construction of DNA cassettes and plasmid vectors To construct the plasmid vector pBB4H1, the human H1 promoter was PCR amplified from human genomic DNA using the following primers: forward 5'-CCATGGAATTCGAACGCTGACGT-3' and reverse 5'-GCAAGCTTTGGTCTCATAAGAACTTATAAGATTCCC-3', which contain one *Eco*RI and one *Hin*dIII restriction site, respectively. The PCR product was subcloned in the pDRIVE vector (Qiagen, Valencia, CA) following the manufacturer's instructions. The pDRIVE-H1 vector was then digested with *Eco*RI and *Hin*dIII to obtain a DNA fragment of ~230 base pairs, which contained the H1 promoter. This fragment was then cloned into the EcoRI and *Hin*dIII site of the pBlueBac4 vector (Invitrogen, Carlsbad, CA) to obtain the pBB4H1 vector.

To generate the DNA cassettes the following chemically synthesized oligonucleotides were used: hpGFP1 5'-GCAAGCTTCCCCAAA*AACCACTACCTGAGCACCCA*GGGGCCCC-3', hpGFP2 5'-GCAAGCTTCCCCAAA*AAGGGCGAGGAGCTGTTCACCGG*GGCCCC-3', hpGFP3 5'-GCAAGCTTCCCCAAA*AACGGCCACCAGTTCAGCGT*GGGGCCCC-3', hpGFP4 5'-GCAAGCTTCCCCAAA*AAGGAGGACGGCAACATCCT*GGGGCCCC-3', hpTRPC4 5'-GCAAGCTTCCCCAA*AAAUUACUCGUCAACAGGCGGACGG*GGCCCC-3'. Note that the sequence upstream of the 5 A's contained the *Hin*dIII restriction site (underlined), followed by 4 C's, and was included in the oligonucleotide design because it allows easy cloning of the DNA cassette and allows the RNA pol III to initiate in the first of the 5 A's, exactly 30 nucleotides after the TATA box. The sequence in italics corresponds to the *eGFP* mRNA.

To construct the DNA cassettes, 10 μL of each oligonucleotide (~35 μmol/L) were heated for 5 min to 94 °C. When the temperature reached 40 °C, 10 μL of a reaction mix was added [reaction mix: 2 mmol/L dNTPs; 5 U Klenow enzyme (Invitrogen); 2 μL 10×reaction buffer 2 (Invitrogen); 10% Me_2SO; H_2O to 10 μL]. All the reactions were performed identically using a thermo-cycler to precisely control the temperatures (Figure 1).

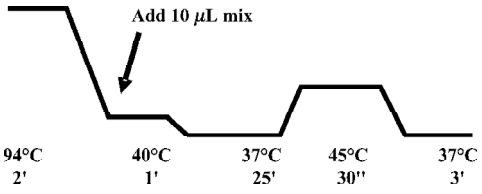

Figure 1. Temperature sequence used in the thermocycler reaction for the construction of DNA cassettes.

Then, the Klenow products were heated to 65 °C to inactivate the enzyme, digested with *Hin*dIII and cloned into the *Hin*dIII restriction site of the pBB4H 1 vector previously dephosphorylated with alkaline phosphatase (Roche, Basel Switzerland). Only the correct cassettes would be ligated into the *Hin*dIII cohesive ends. We did not observe clones with tandem-repeated cassettes.

The sequences targeting the *eGFP* mRNA were chosen based on 2 criteria: (i) because they had fewer than 3 G or C repeats to prevent G-quartet formation; and (ii) because they were distributed along the *eGFP* mRNA.

Cell culture and transfection HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and antibiotics, and were seeded every 3 d following standard procedures at 37 °C. The day before transfection, cells were grown without antibiotics in 12 well plates. Vectors containing the DNA cassette were transfected using lipofectamine 2000 (Invitrogen) at different concentrations following the manufacturer's instructions. The day after transfection, the cells were washed once with phosphate-buffered saline (PBS) and treated with 5 MOI of a recombinant adenovirus encoding the green fluorescent protein (eGFP) gene under the transcriptional control of the cytomegalovirus (CMV) promoter[22]. Four hours later, the cells were washed with PBS and the media was replaced. Twenty-four hours later the fluorescence was evaluated using a FACScan flow cytometer (Becton & Dickinson, Franklin Lakes, NJ). To evaluate the fluorescence intensity, 10 000 events were counted in at least 3 independent experiments for each concentration.

Confocal microscopy The HeLa cells that were treated with the plasmids and adenovirus as described were washed with PBS, trypsinized and plated on sterile coverslips.

Twenty-four hours later, the cells were analyzed with a confocal microscope (Bio-Rad, Hercules, CA) using a 40× objective. The confocal images were analyzed using the Confocal Assistant freeware software.

Results

Construction of DNA cassettes For the construction of the DNA sequence that served as a template for shRNA transcription, we followed the principle of DNA amplification commonly used in PCR reactions (Figure 2A). That is, any given sequence that serves as a primer could be amplified at its 3' end with a proper DNA polymerase. In RNAi hairpins, the 2 strands that form the stem are fully complementary, so it is possible to use only a single oligonucleotide that is self-complementary within its 3' end (Figure 2B). After the self-complementary structure is formed, it is possible to further amplify the structure so that the oligonucleotide becomes completely double-stranded DNA.

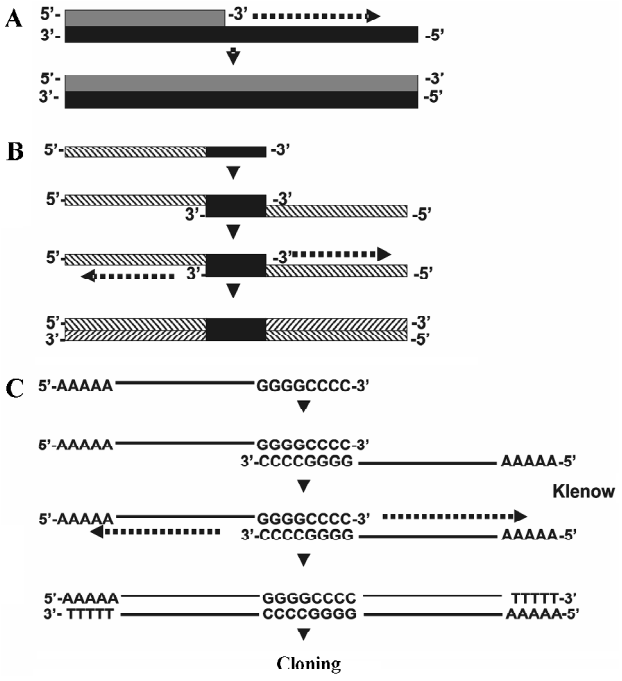

Figure 2. Using the amplification principle (A), an oligonucleotide can be amplified if it is auto- hybridized by its 3' end (B). We selected the sequence 5'-GGGGCCCC-3' to form the secondary structure shown in (C), and we added it to the 21 nucleotide sequence. Also, to obtain the transcription termination, 5 A's are included in the oligonucleotide. After a filling-in reaction by the Klenow enzyme, a DNA cassette is formed. The optimum enzymatic temperature of the Klenow fragment is approximately 37 °C, which corresponds to the T_m value of the 5'-GGGGCCCC-3' sequence. To avoid enzyme denaturation, the Klenow fragment and dNTP mix is added until the temperature drops to 40 ºC (see text, and Figure 1). Temperatures may be automated with a thermocycler.

The self-complementary pairing must be strong enough to allow the DNA polymerase to initiate the reaction at the selected temperature. We chose the sequence 5'-GGGGCCCC-3' due to its high T_m (37 °C) and because its palindrome nature permits the self-complementary structure. Furthermore, this sequence allows amplification using the large (Klenow) fragment of the DNA polymerase, whose working temperature is 37 °C. Thus, because the 5'-GGGGCCCC-3' sequence is amplified and lies between the sense and antisense strand, it also functions as a loop for the shRNA (Figure 4).

Finally, most RNAi vectors contain an RNA pol III promoter such as U6 or H1, which is used to initiate the transcription of the shRNA. When the RNA pol III finds 5 consecutive T's, it terminates the transcription and removes the last three nucleotides on the 3' end[17, 20]. Therefore, we included 5 A's in the 5' end in the oligonucleotide; when the Klenow fragment synthesizes the antisense strand, it generated the 5 T's, which will be needed to terminate the transcription by RNA pol III inside the cell (Figure 2C). The expected shRNA structures that will form inside the cells are shown in Figure 4. All these structures were modeled using the Mfold server (see Materials and methods).

To determine if it was possible to construct the DNA cassette for shRNA vectors with this method, we designed and tested its ability to amplify different oligonucleotides. These oligonucleotides were prepared to target sequences from *eGFP* and a control oligonucletide containing an eGFP-unrelated sequence. eGFP oligonucleotide sequences were selected to have low GC repeats to prevent G-quartet formation. Figure 3 illustrates the result of a typical filling-in reaction, showing that the Klenow fragment produced double-stranded DNA from single oligonucleotides. Therefore, the self-complementary 5'-GGGGCCCC-3' sequence is a good template for DNA amplification. Furthermore, all cassettes were constructed simultaneously in a single step reaction, so the 1-oligonucleotide method could be automated to perform a large number of reactions in a short period of time (see Materials and methods and Figure 1). We cloned the DNA cassettes in the vector under the transcriptional control of an RNA pol III (H1) promoter, and all cassettes were sequenced to confirm their integrity.

Efficacy of the 1-oligonucleotide method in RNAi experiments To test the ability of the DNA cassettes to trigger RNAi activity, HeLa cells were transfected with the plasmid vectors containing the cassettes targeting the mRNA from eGFP and a control construct. The day after transfection, cells were incubated with a recombinant adenovirus encoding the eGFP, and 24 h later the cell fluorescence was

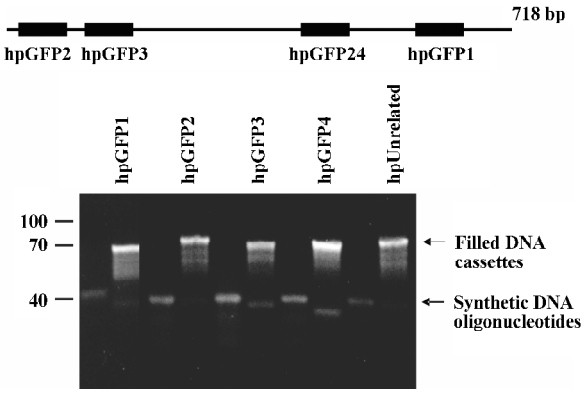

Figure 3. Automation of the method. Using the same conditions as described in the text, a wide variety of DNA cassettes were generated simultaneously. A low melting point 4% agarose gel (*w/v*) is shown in the bottom half of the figure, with the filled shDNAs and control oligonucleotides (controls are on the left of the shDNA); the expected length of each filled DNA cassette is ~75 bp. The 21 nucleotide sequences correspond to the enhanced eGFP mRNA. The target positions of hairpin GFP1–4 (represented by black boxes) are shown on top: hpGFP1, nucleotides 595 to 616; hpGFP2 nucleotides 10 to 31; hpGFP3, nucleotides 69 to 90; hpGFP4, nucleotides 394 to 415; the length of the eGFP sequence is 718 bp. After the Klenow filling-in reaction, DNA cassettes were cloned into an RNA pol III vector for transfection.

evaluated. All the tested sequences silenced eGFP expression and, as expected, there was no gene silencing with the transfection of the unrelated construct (Figure 4). A dose–response experiment showed that the most effective plasmid concentration to achieve RNAi was 1–3.75 μg/mL. Of 4 tested DNA cassette sequences (termed hpGFP1 to 4), hpGFP 2, 3 and 4 inhibited eGFP expression/fluorescence by more than 70%. The less efficient cassette (hpGFP1) inhibited eGFP expression by 50% at 1 μg/mL (Figure 5). The EC_{50} of each shRNA confirms this result; the less effective shRNA is hpGFP1, with an EC_{50} of approximately 0.5 μg/mL, whereas hpGFP 2–4 have EC_{50} values of approximately 0.05 μg/mL (Figure 5). The finding that not all DNA cassette sequences had the same potency confirms the fact that a variety of sequences must be designed for the same target in order to find the most effective RNAi inducer. However, it was surprising that the hpGFP 1 sequence was the least effective of all tested sequences. This result was interesting because this sequence had been shown to be a strong inhibitor of eGFP expression in a previous study[23]. The dose-response experiment with the unrelated cassette did not influence the eGFP fluorescence (data not shown). Together, these results suggest that diverse DNA cassettes must be produced by the 1-oligonucleotide method in order to identify the best sequence for RNAi.

Discussion

In the present work we developed a novel, easy and efficient 1-oligonucleotide method to generate DNA cassettes for RNAi vectors. To test the capability of the method, we designed DNA cassettes to target the mRNA from eGFP. For this purpose a recombinant adenovirus encoding the eGFP gene was used to infect HeLa cells. Only cells infected with the virus and transfected with specific anti-GFP cassettes strongly inhibited eGFP fluorescence. The level of inhibition achieved with this method resembles the inhibition when RNAi is used to knock-down the expression of endogenous genes, because transfections usually reached 60%–75% of the cells, and the level of adenovirus infection almost reached 100%. The results obtained here suggest that eGFP expression was strongly reduced in the cells that were transfected with the DNA constructs, as illustrated in Figure 5. The fact that hpGFP1 was less efficient at knocking down eGFP expression suggests that a variety of RNA sequences are needed in order to find the most effective. Although this sequence has already been used by others[23], it is reasonable that there could exist sequences that are even more effective; this method is an easy way to construct a large variety of DNA cassettes for 1 or more genes. Together, these results demonstrate that, although eGFP expression is under the transcriptional control of the strong CMV promoter in the adenoviral vector, it is still possible to induce gene silencing using the cassettes described here.

Previously reported methods to produce RNAi using DNA cassettes to generate shRNAs in mammalian cells require long oligonucleotides or several PCR reactions that are sequence and step specific[17,20,21,24–28]. For example, in the method reported by Gou et al, at least three oligonucleotides (1 forward and 2 reverse) are used in 2 sequential PCR reactions to generate the DNA cassette[21]. Once the 2 PCR reactions conclude, PCR products require further cleaning before transfection into cells. Another method previously reported is based on primer extension, and the DNA cassettes are cleaned and introduced in the cells directly[26]. Although the latter method uses only 2 oligonucleotides to construct each DNA cassette, these oligonucleotides are fairly long (approximately 100 nucleotides), considerably increasing the cost of constructing multiple cassettes.

There are a number of effective methods that have been designed over the past few years to produce RNAi in mammalian cells[29], but unfortunately none of these methods is easily automated because they require many step-specific reactions. Using the method described here, we simulta-

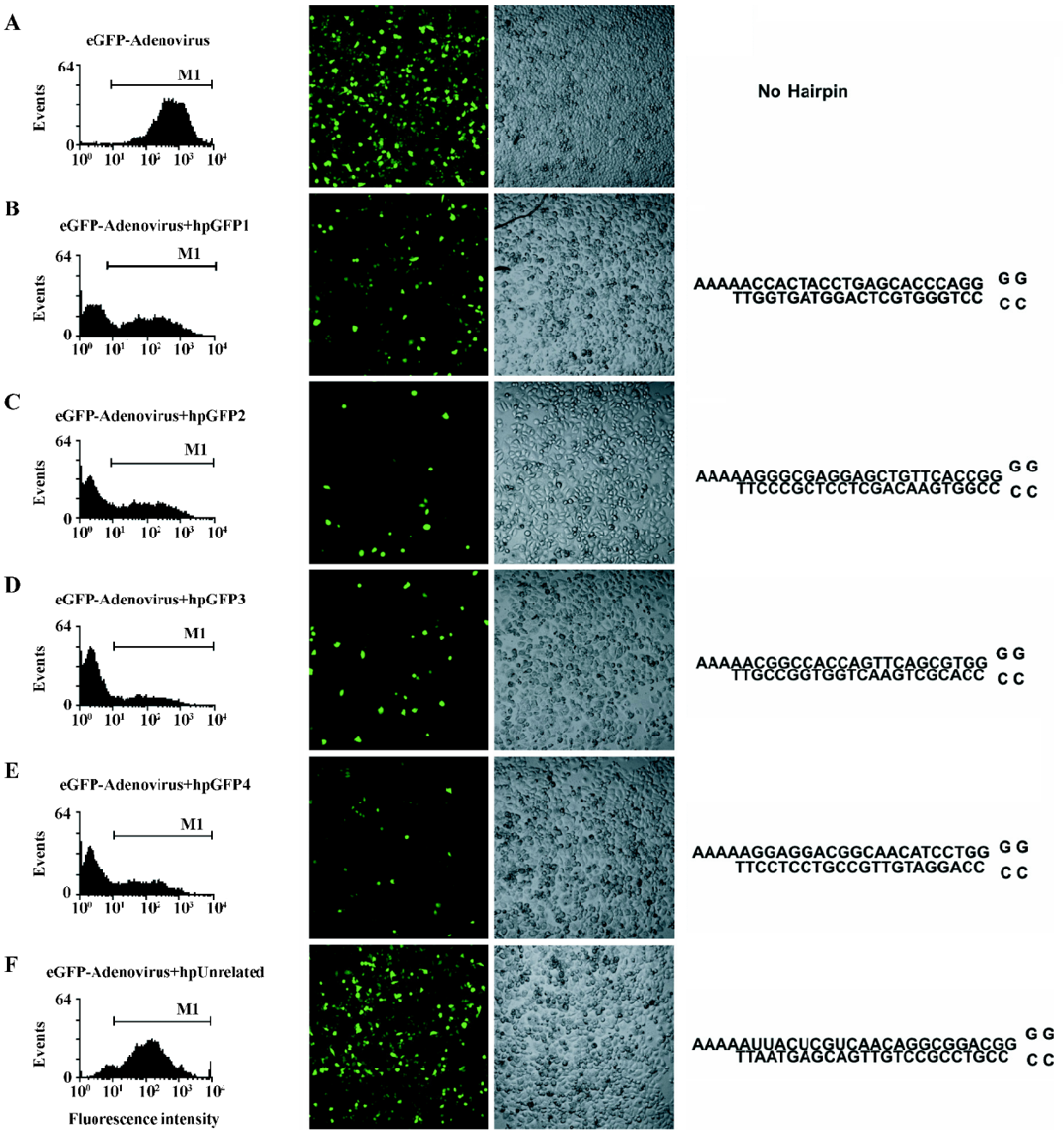

Figure 4. Inhibition of eGFP by plasmids that produce shRNAs. HeLa cells were transfected with 3.5 mg/mL of plasmid DNA. Confluent cells (~80%) were infected 24 h later with 5 MOI of a recombinant eGFP-adenovirus, and the next day the fluorescence was evaluated by confocal microscopy and flow cytometry. Left panels: Representative experiments measured by flow cytometry; the region marked M1 was considered to be the cell population expressing eGFP. Middle panels: Confocal images showing eGFP-positive cells, and the transmitted light shows the total number of cells in the field. Right panels: shRNA predicted structures. The shRNA structures were modeled based on the sequences that should be transcribed inside the cell after plasmid transfection. The shRNAs were modeled with the Mfold server: http://www.bioinfo.rpi.edu/applications/mfold/, and the conditions were 37 °C and 2 mmol/L Mg^{2+}.

neously produced a large number of different DNA cassettes targeting different mRNAs (unpublished data). This is possible because the process can be automated to perform many different reactions using the same temperature, time and reactant conditions using a thermocycler.

The advantages of the method described here over pre-

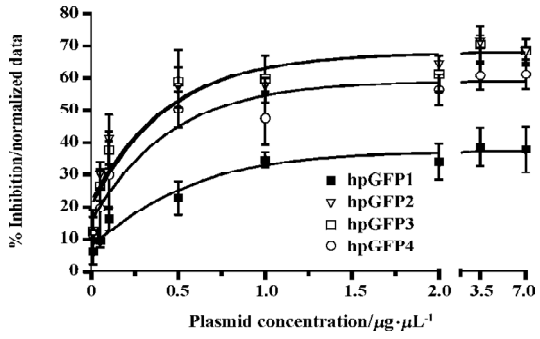

Figure 5. Quantification of eGFP fluorescence by flow cytometry. Data from all inhibition experiments were fitted to a dose-response equation (actual fit represented by the solid lines), showing that different cassettes have different RNAi potencies. Data were analyzed by using the GraphPad Prism 4.0 software. Data were obtained from at least three independent experiments and were mean±SEM.

viously reported methods are: (i) a single oligonucleotide is sufficient to generate a DNA cassette for shRNA production, significantly reducing the price of testing multiple sequences; (ii) oligonucleotides for cassette construction are easy to design: a 21–25 nucleotide sense target sequence is flanked by 5'-AAAAA- and -GGGGCCCC-3', thus the length of the oligonucleotide is considerably shorter; and (iii) the method is automated, allowing the construction of a wide variety of DNA cassettes in one step in a few minutes.

Recently it was shown that it is also possible to express synthetic miRNAs using RNA pol II promoters, which allows still more control over stem-loop RNA expression, because many RNA pol II promoters function in a inducible/tissue-specific fashion[30]. We are currently constructing DNA cassettes by using the 1-oligonucleotide method for use with RNA pol II promoters (unpublished results). Because this type of polymerase does not terminate the transcription after 5 consecutive thymidines it is not necessary to include 5 adenines in the oligonucleotide, which further reduces the length and price of the DNA cassette.

In conclusion, we reported here a novel method for constructing large libraries of RNAi cassettes quickly and in a cost-effective manner, thus making the exploration of the function of a large number of genes an easier task and facilitating functional genomic studies.

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