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Generation of shRNA Pool Library: A Revision of the Biological Technique from the Viewpoint of Chemistry

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RNA interference (RNAi) is the process of using a small double-stranded RNA (siRNA) to knock down the expression level of sequence–homologue genes.[1] The selective and robust effect of siRNA on gene expression makes RNAi one of the most important technological breakthroughs in modern scientific history.[2] Short hairpin siRNA (shRNA) is the most widely used form of expressed siRNA in which RNA duplexes are tethered through a small loop.[3] Currently the use of individual shRNA to study gene function is commonplace. The newest trend is to use siRNA/shRNA libraries to perform reverse-genetic screens of hundreds or even thousands of different genes in a single experiment.[2a]

Three major factors limit the broad application of synthetic-array-based siRNA libraries: the enormous cost of genome-wide siRNA libraries and the expensive robotic liquid handling systems for high-throughput screening as well as the difficulties in performing transfections reproducibly.[2a] A much easier alternative is to prepare an siRNA library that is first expressed in transduced cells, then the cells that have the desired phenotype are selected, and the siRNA sequence that is contained within the selected cells is identified. Several groups have reported a technique for the construction of such a shRNA pool library.[4] The key step in this technique is to convert a pool of single-stranded hairpin DNA into a pool of double-stranded DNA (shRNA templates) via special DNA polymerase-catalyzed primer extension (Figure 1 A) prior to cloning into expression vectors (see Figure S1 in the Supporting Information). Given the enormous advantage of this method, however, no follow-up application has been reported. Here, we revise the feasibility of this step from the viewpoint of chemistry.

The goal of primer extension is to disrupt intramolecular base pairs within the highly stable hairpin DNA template, and to synthesize a complementary single-stranded DNA as shown in Figure 1 A. It should be noted that the newly synthesized single-stranded DNA is not only complementary, but more importantly, sequence-identical to its template, and thus their annealing product is a palindromic DNA, except for the small loop portion. However, the fundamental concern is whether this reaction, that is, the displacement of intramolecular base pairs with intermolecular base pairs, is energetically favorable, or which form in this conversion, hairpin or the double strand, is more stable.

In order to clarify this point, we tested the thermostability of palindromic DNA. A pool of double-stranded DNA (the siRNA-coding sequences within this pool are completely randomized, see below) was heated at 50 °C or 90 °C for 5 min and then cooled to room temperature (25 °C) or left at 25 °C for one month. As shown in Figure 1 B, more than half of the double-stranded DNA transforms into hairpin DNA when heated at 50 °C, and this trend becomes more evident at 90 °C—all of the DNA exists in a hairpin form. These results strongly suggest that the palindromic DNA is less stable than its constituent hairpin DNA (Figure 1 C) and cannot be prepared by routine

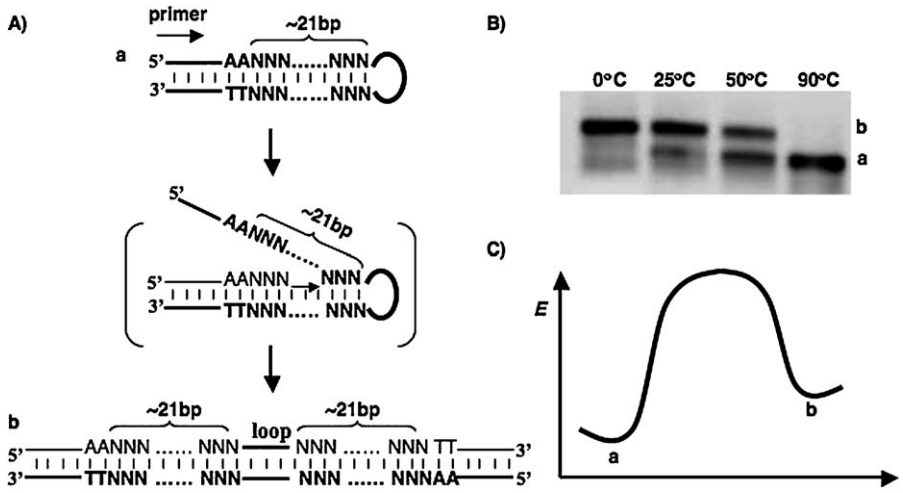

Figure 1. A) Schematic of the reported protocol for the conversion of a pool of hairpin DNA (a) into a pool of palindromic dsDNA (b) via primer extension. B) Thermostability of the palindromic dsDNA (b) monitored by 2% agrose gel. C) Generic potential energy diagram of the hairpin DNA (a) and the palindromic dsDNA (b) in the reaction of primer extension.

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DNA denaturing and annealing. The thermostability test also suggests that conversion of a palindromic DNA from a double-stranded form into a hairpin form is a spontaneous reaction; this is supported by the slow conversion at room temperature. This is reasonable from the viewpoint of chemistry because intramolecular base-pair formation (hairpin DNA) is always faster and more efficient than intermolecular base pair formation (double-stranded DNA).[5] Therefore, the step in the reported biological technique, that is, the conversion of hairpin DNA into double-stranded DNA, is unlikely to be feasible from a thermodynamical point of view, and experimentally it proved to be inefficient in our hands.[6]

Primer extension is a common method that is used in DNA sequencing, and self-ceasing is an intrinsic obstacle for sequencing long palindromic DNA;[7] this hurdle was encountered in the currently reported technique. The role of DNA polymerase in primer extension is to react with dNTP to form an intermediate that subsequently condenses with the 3′-hydroxyl group at the end of the extending DNA, to form a new base pair with the template.[8] Although special DNA polymerases, such as the reported Bst or phi29, which have the ability of strand displacement[4] might provide an alternative route to products, as catalysts they cannot make energetically unfavorable reactions possible. In chemistry and biology, catalysts have no effect on the chemical equilibrium of a reaction because the rate of both the forward and the reverse reaction are equally affected. The net free energy change of a reaction is the same whether a catalyst is used or not.[9] Therefore, if the equilibrium is greatly displaced in one direction, the reaction is effectively irreversible and the enzyme only catalyzes the reaction in the thermodynamically allowed direction. In this case, the preparation of palindromic DNA from hairpin DNA is clearly in the unfavorable direction; this cannot be altered by any enzyme.

Here, we report an approach that converts the impossible reaction of primer extension into a thermodynamically favorable one. The scheme of our approach and experimental results are shown in Figure 2 A and B, including 5 steps: 1) anneal a partial randomized hairpin DNA (oligo **1**) to a hairpin primer (oligo **2**), wherein the 5′-end of the oligo **2** is annealed to the 5′ end of oligo **1** with its terminal base mismatched (Figure S2); 2) extend the 3′-ends of the obtained oligo complex **3** by the Klenow fragment to form an open dual-hairpin (oligos **4**) with an unligated gap between the extended 3′-end of oligo **2** and the 5′-end of the oligo **1**, and a mismatched flap between the extended 3′-end of oligo **2** and the 5′-end of oligo **2**; 3) ligate the gap in the dual-hairpin structure **4** by T4 ligase to form a longer single-stranded DNA **5**; 4) extend the 3′-end of the long DNA along itself by a DNA polymerase to form a more stable and longer hairpin DNA **6**; 5) cleave the long hairpin DNA by the restriction enzyme BamHI at low temperature (37 °C) to release the double-stranded palindromic DNA. The sequence of palindromic DNA is confirmed by sequencing when ligated into shRNA expression vectors (Figure S3).

In this approach we utilize a hairpin primer that is ligated with the siRNA-coding hairpin DNA template to form an open dual-hairpin DNA (**5**). Introduction of the second loop converts the unlikely primer extension in the original intramolecular base-pairing reaction into a kinetically favorable intramolecular primer extension. The product from this intramolecular reaction is a single hairpin DNA that contains the palindromic dsDNA segment rather than an unstable palindromic dsDNA. The added loop gives the palindromic product more thermostablility than the corresponding hairpin DNA template, and thus the extra energy upon the reaction drives the equilibrium

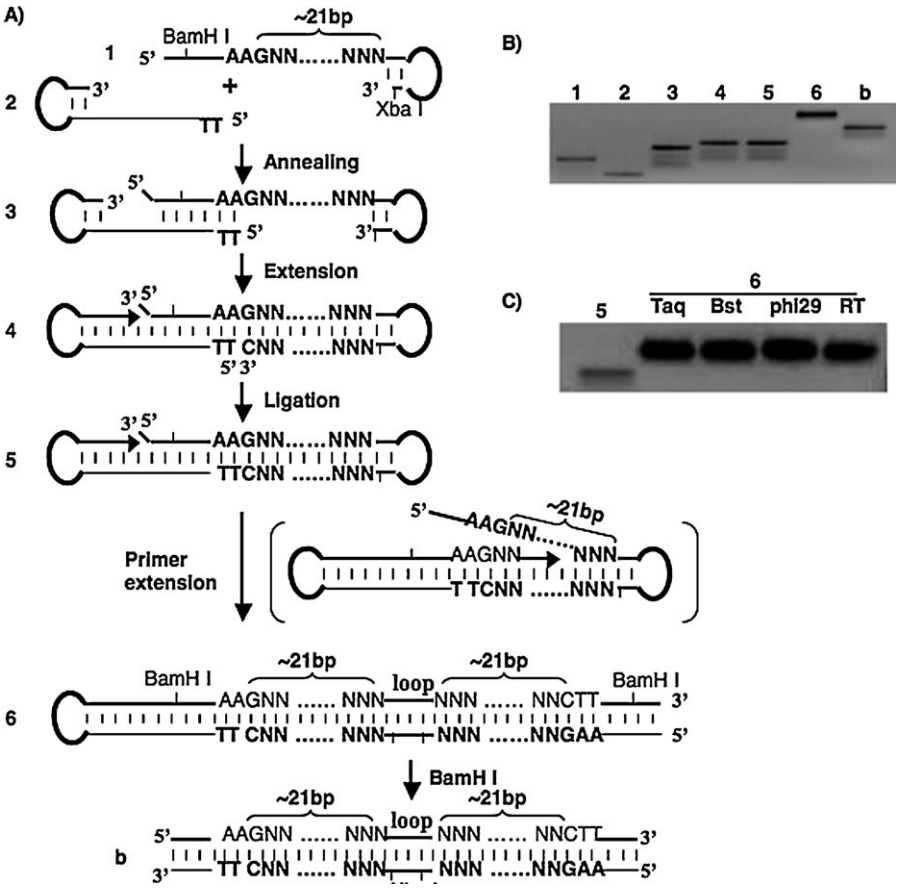

Figure 2. A) Schematic outline of the present approach for the 5-step conversion of a pool of hairpin DNA into a pool of palindromic DNA. B) PAGE of the DNA products during the course of reactions. C) Primer extension catalyzed by different enzymes.

to the opening of a highly stable hairpin DNA template; this makes a significant contribution to the primer extension. The first four steps in our approach can be combined into a one-pot reaction without purification or separate processing, and the sequence of the hairpin loop can be further optimized.[10] We tested four different polymerases for primer extension, including the reported Bst and phi, and found that all enzymes work efficiently as shown Figure 2C. It should be noted that once the palindromic DNA is produced, it should be immediately ligated into a shRNA expression vector or stocked at low temperature to prevent the automatic conversion into hairpin DNA.

In summary, the reported biological technique for shRNA library construction is unlikely to be feasible from the viewpoint of chemistry, and this issue is addressed by converting an intermolecular primer extension into an intramolecular extension. This new approach will enable most labs and researchers to generate shRNA pool library, which can be used to perform reverse-genetic screens for genome-wide evaluation of human genes.

Experimental Section

Please refer to the Supporting Information for a more detailed description of the oligo sequences. The typical procedure for oligo denaturing, annealing, and extension is described as following: a mixture of oligos **1** and **2** in a molar ratio of 1:1 in the corresponding DNA polymerase buffer was heated at 95 °C for 10 min and then gradually cooled to room temperature. The various DNA polymerase plus 200 μM dNTP were added, and the solution was heated to the required temperature for primer extension (72 °C for Taq, 65 °C for Bst, 30 °C for phi29 and Klenow Fragment).

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