Enzymatically prepared RNAi libraries

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Large-scale RNA interference (RNAi) screens in mammalian cells have mainly used synthetic small interfering RNA (siRNA) or short hairpin RNA (shRNA) libraries. The RNAi triggers for both of these approaches were designed with algorithm-based predictions to identify single sequences for mRNA knockdown. Alternatives to these approaches have recently been developed using enzymatic methods. Here we describe the concepts of enzymatically prepared shRNA and siRNA libraries, and discuss their strengths and limitations.

The discovery that long double-stranded RNA (dsRNA), as used for gene silencing in worms and flies, is processed by the enzyme Dicer into siRNAs[1,2] has extended RNAi to mammalian cells[3]. Since then, several siRNA and shRNA libraries have been generated for large-scale functional studies, and they are commercially available from several suppliers[4–6]. Both siRNA and shRNA libraries require the synthesis of either RNA or DNA oligonucleotides that are transfected directly or first cloned into an expression vector, respectively. Therefore, good *a priori* prediction of the silencing efficiency is required to obtain a molecule with high silencing efficiency and specificity[7,8]. In contrast to these approaches, which rely on knowledge-based silencer design, enzymatic methods use cDNA as starting material and yield a large number of different silencers in one step. This pool of silencers presents an efficient and specific alternative to synthetic libraries (**Table 1**).

Enzymatically prepared siRNA libraries

Chemically synthesized siRNAs are the most commonly used and well-characterized mediators for RNAi in mammalian cells. But they suffer from two drawbacks that may hamper their use for large-scale studies in particular: the variable inhibitory abilities of different siRNAs and the high cost of synthesis[9]. Because of the variability, each siRNA must be screened for its silencing efficiency, or more than one siRNA per gene has to be used to ensure efficient gene silencing. This feature, in combination with the cost issue, limits the use of chemically synthesized siRNAs in large-scale studies for most academic laboratories.

Enzymatic strategies. We and others have pioneered a fast and cost-efficient technology to circumvent these problems by preparing siRNA through digestion of long dsRNA with endoribonucleases *in vitro*[10–14]. By using bacterial RNase III, which can be easily expressed and purified from *Escherichia coli,* or recombinant Dicer (both enzymes are also commercially available), long dsRNA can be converted *in vitro* into siRNAs with a length of 18–25 base pairs (bp). This process generates a heterogeneous population of siRNAs capable of interacting with multiple sites on the target mRNA, and recapitulates the potent and specific mRNA knockdown by long dsRNA in worms and flies. The resulting endoribonuclease-prepared siRNAs (esiRNAs) or diced siRNAs (d-siRNAs) have been proven to knock down genes efficiently in mammalian tissue culture cells[10,15–18], and in the developing[19,20] and adult mouse[21], and to inhibit viral replication[22]. For these reasons and because of the simplicity, cost-effectiveness and time-efficiency of the synthesis, esiRNA technology appears to be a versatile alternative to chemically synthesized siRNA for RNAi studies in mammalian cells.

For the generation of large-scale esiRNA libraries, we have developed a robust and simple protocol. A cDNA fragment tagged with T7 promoter sequence by PCR is transcribed to produce dsRNA *in vitro*. By limited digestion, the long dsRNA is then converted to siRNAs less then 30 bp in length, and subsequently isolated by spin purification in a single column[23] (**Fig. 1**). This method allows the very rapid generation of esiRNAs (>1,000 per day) for genome-wide

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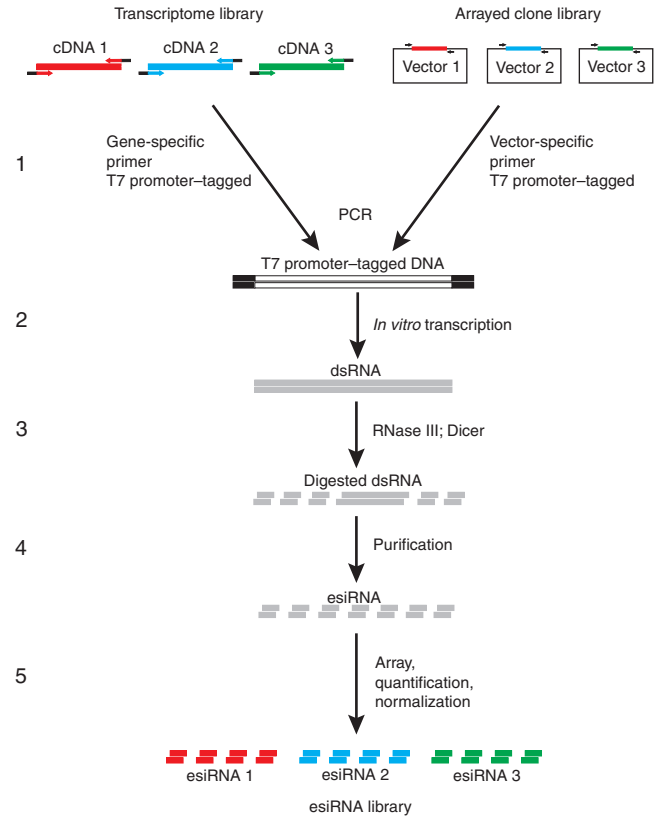

Figure 1 | Generation of siRNA pools by endoribonuclease cleavage. The source of DNA can be either a cDNA library or a library of plasmids carrying the DNA of interest. DNA is PCR-amplified with either gene-specific primers or primers complementary to the plasmid backbone (Step 1). In both cases primers contain T7 promoter sequences. This allows the *in vitro* transcription (Step 2) with T7 RNA polymerase to generate long dsRNAs. Digestion with either RNase III or Dicer results in a pool of short dsRNA molecules (esiRNAs; Step 3). Purification by ion exchange chromatography removes undigested dsRNA to yield the final pool of functionally sized esiRNAs (Step 4). Adjustment to the same concentration and arraying in a format feasible for high-throughput applications (Step 5).

libraries in a standard-sized academic laboratory. Similar protocols have been developed for the generation of diced siRNAs[24].

Applications. When applying this strategy for the first generation of human and mouse esiRNA libraries, we have used cDNA clones, which have the advantage that universal vector-specific primers can be used to amplify the cDNA insert. By using sequence-verified sets of cDNA clones, we have generated libraries of ~15,000 human esiRNAs and ~25,000 mouse esiRNAs. Human esiRNA libraries have recently been used in large-scale screens to identify previously unknown genes required for cell division in human cells[25] and new calcium sensors[26], highlighting the potential of enzymatically prepared siRNA libraries for large-scale screens.

Future improvements. Although cloned cDNA libraries have proven to be a good resource for esiRNA library generation, we anticipate that the library quality can be improved. The prediction algorithms for the design of efficient siRNAs and shRNAs have been refined with the growing mechanistic understanding of RNAi. These algorithms have been implemented into software programs such as DEQOR[27] to allow automatic analyses of transcripts. These programs can be used to scan all protein-coding transcripts of sequenced transcriptomes for the identification of the best esiRNA targeting regions. To obtain the regions with highest density of good silencers, we have designed target-specific primers appended with T7 promoter sequences, which allows the amplification of *in silico* predicted targeting regions with a length of 400–600 bp for esiRNA production. These primers will be used to create second-generation esiRNA libraries targeting all protein-coding genes for human, mouse and rat. The PCR template library can be easily reamplified with one universal T7 primer, and will be made available as an unlimited resource to the scientific community.

Enzymatically prepared shRNA libraries

Three groups independently reported the generation of enzymatically prepared shRNA libraries referred to as EPRIL[28] (enzymatic production of RNAi library), REGS[29] (restriction enzyme-generated siRNA) and SPEED[30] (siRNA production by enzymatic engineering of DNA). These approaches all have a similar principle but differ in details of their production (**Fig. 2**).

Enzymatic strategies. All three methods use cDNA as the starting material, which is digested into small fragments using different protocols. In the EPRIL method, DNase I digestion is used to generate fragments of ~100–200 bp. For the REGS and SPEED methods, a mixture of restriction enzymes that recognize abundant sequences and generate 5′C-G overhangs is used. A palindromic structure of the insert is necessary for creating a hairpin structure to form the shRNA. To achieve this, the fragments are ligated to hairpin-DNA containing an *Mme*I restriction site. *Mme*I cuts 3′ from its recognition site, generating fragments with sizes in the range of those of siRNAs. After the *Mme*I digestion, the products are ligated to a second DNA adapter. For REGS, the second linker is also a hairpin. Ligation products are amplified by Φ29 DNA polymerase in a rolling-circle amplification, followed by digestion of the DNA concatemers. For EPRIL and SPEED, linear dsDNA adaptors are ligated, and Bst DNA polymerase is applied for primer extension. Both Φ29 and Bst DNA polymerases have strand-displacement activity, which is a prerequisite for polymerization through a template with a stable secondary structure. Finally, in all methods, the product is digested with restriction enzymes and subcloned into a plasmid downstream of a polymerase III promoter. As a variation of these methods to produce shRNA libraries, Seyhan and colleagues have used cloned Dicer or RNase III products to generate siRNA expression libraries using opposing U6 and H1 promoters[31].

Applications. Enzymatically prepared shRNA libraries are advantageous because of their flexibility. First, they can be used to identify an optimal shRNA for a single gene. Using the full-length cDNA of a gene of interest as the starting material ensures that the whole sequence space is explored experimentally to find the best silencing trigger. To identify the best shRNA from the pool quickly, Zhao and colleagues recently developed an elegant assay using bacterial invasion as the shRNA delivery method[32].

Second, these libraries represent a very large pool of shRNAs targeting the entire transcriptome, when a complex cDNA mixture is used as the starting material. Hence, these libraries should be useful for positive selection screens. Ideally, a normalized cDNA library

should be used to avoid having highly expressed genes overrepresented in such a library. The largest library reported so far contains about three million clones[30], and is therefore more complex than any of the synthetically prepared shRNA libraries. Notably, some of the identified efficient silencers did not match the rules of the prediction algorithms used to design synthetic shRNAs. This suggests that evaluation of the results obtained with the enzymatic shRNAs may help to refine the prediction algorithms.

Third, these methods could be used to generate arrayed complex shRNA libraries for single transcripts. This could be achieved by arraying specific cDNAs, which are converted into enzymatically prepared shRNA pools in a multiwell format. This strategy would allow gene-by-gene screens with multiple shRNAs targeting the same gene. Finally, enzymatically prepared shRNA libraries allow flexible delivery into cells, including the delivery of the silencing trigger via viral infection[28–30].

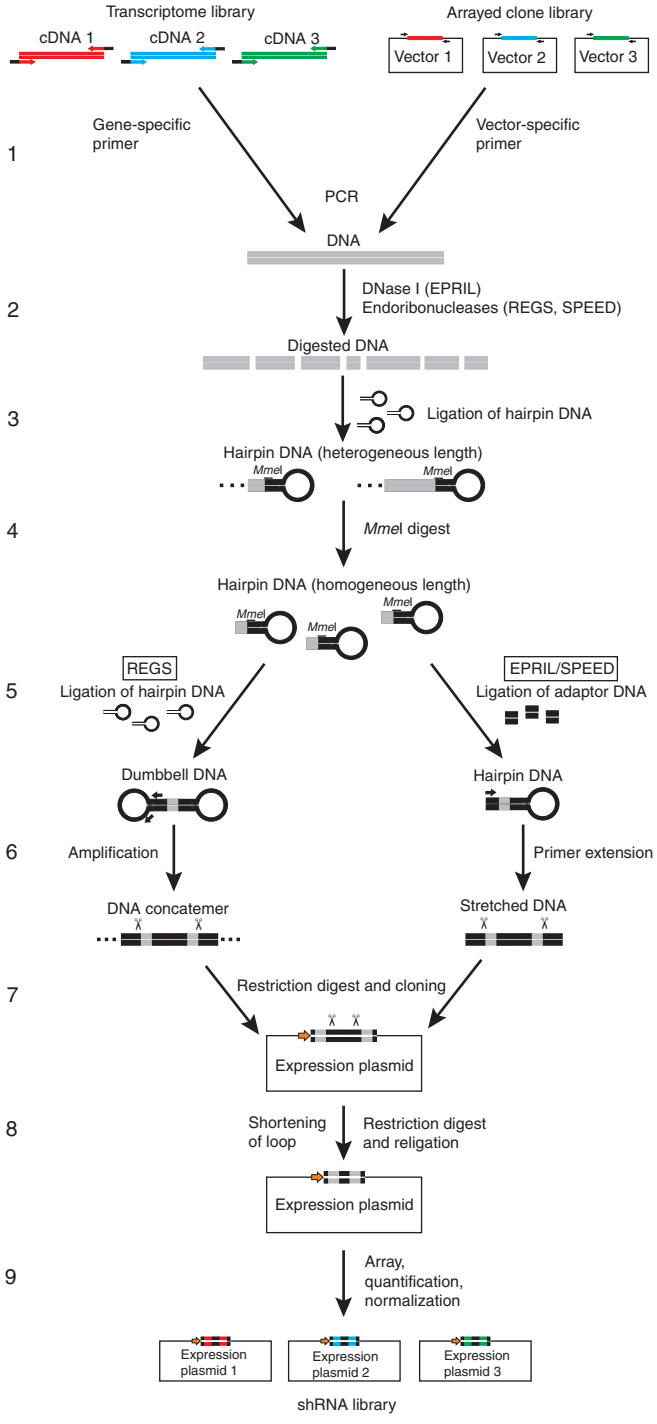

Figure 2 | Construction of shRNA libraries using the methods EPRIL, SPEED and REGS. The source of DNA can be either a cDNA library or a library of plasmids carrying the DNA of interest. DNA is PCR-amplified with either gene- or vector-specific primers (Step 1). DNA is fragmented using either a mixture of restriction enzymes (REGS, SPEED) or is randomly cut by DNase I (EPRIL; Step 2). Hairpin adaptors containing an *Mme*I recognition site are ligated to the fragmented DNA to link sense and antisense strands (Step 3). Note that the hairpin linker can be ligated to either one or both termini of the DNA fragment thereby generating a dumbbell structure or an elongated hairpin, respectively (indicated by black dots). Restriction digest by *Mme*I generates an uniformly sized pool of extended hairpins (Step 4). *Mme*I cleaves 20 bp 3′ from its recognition sequence. Ligation of a hairpin linker (REGS) to the extended hairpin results in the formation of a dumbbell structure (Step 5). EPRIL and SPEED preserve the hairpin structure by ligation of linear dsDNA adaptors. Conversion into a palindromic dsDNA is performed by rolling circle amplification (REGS) or primer extension (EPRIL, SPEED; Step 6). Thereby a concatemer DNA (REGS) or a stretched dsDNA (EPRIL, SPEED) is formed. Restriction and subcloning into an appropriate expression plasmid (Step 7). Digestion of the subcloned DNA within the loop region eliminates extraneous sequences, and religation forms the final expression plasmid (Step 8). Expression plasmids encoding shRNAs against different target genes are arrayed in a format feasible for high-throughput applications (Step 9).

Future improvements. Despite these advantages, there are some limitations. In particular, enzymatically prepared shRNAs have the disadvantage that the methods produce shRNAs originating from the sense- and anti-sense strand of the cDNA. shRNAs from the anti-sense strand will not contribute to the silencing of the gene of interest and, additionally, may silence unintended transcripts. In the long run, a modification of the described methods such that shRNAs are produced only from the sense strand would be desirable.

Enzymatically prepared versus synthetic RNAi libraries

Synthetic libraries consisting of siRNAs or shRNA expression vectors represent selected and defined sequences of the silencing triggers. In contrast, for enzymatically prepared libraries the sequence of the fragment used to create the pool of siRNA or shRNA vectors can be defined, while the identity and abundance (in the case of shRNA vectors) of each individual silencing trigger in the library is unknown. But the design of synthetic libraries requires the knowledge of all mRNA sequences of an organism, whereas enzymatically prepared libraries require only cDNA preparations or clones and could therefore be generated for organisms with unknown genome sequences. Furthermore, enzymatically prepared libraries could also be generated from substractive cDNA libraries or cDNA libraries from a cell after a specific treatment to enrich the shRNA or siRNA library for triggers specific to a given cellular status or pathway.

A major advantage of enzymatically prepared RNAi libraries is their relatively low price. Specifically, the price tag for chemically synthesized siRNA libraries is still too high for most academic laboratories. In contrast, the affordable reagents for the enzymatic production of RNAi libraries are available in most laboratories. Additionally, libraries can be generated quite rapidly. We now can prepare >1,000 esiRNAs per day in our standard-sized laboratory.

The effort to prepare such a library, however, is still considerable. A limited number of esiRNA resources are already available from several suppliers (RZPD, New England BioLabs and Ambion), whereas none of the enzymatically prepared shRNA libraries are commercially available, yet. An important step forward for large enzymatically prepared RNAi libraries would be their commercialization to make them available for those who do not want to prepare them in the laboratory.

Finally, the use of complex mixtures of siRNAs or shRNAs reduces the number of false positives in large-scale studies. Recent publications reported off-target effects for individual siRNAs, challenging the reliability of RNAi data[33–36]. Because cross-silencing apparently requires a relatively low level of sequence similarity to the off-target mRNA[37–39], it can be assumed that most siRNAs and shRNAs have an individual cross-silencing signature. Therefore, it appears difficult, if not impossible, to design siRNAs that will not cause off-target effects. Pooling of siRNAs reduces off-target effects while maintaining efficient silencing of the specific target gene (unpublished data and refs. 24,33,40,41). The explanation for this observation is the lower concentration of individual siRNAs in a pool; because each single siRNA in a pool has different off-targets while having the same on-target, the use of siRNA pools dilutes out off-target effects. Because esiRNAs represent hundreds of different silencing triggers, they exhibit a reduced risk for cross-silencing, as long as highly conserved mRNA regions are avoided as target sites. Sequences with lower conservation are however tolerated. We recently demonstrated that esiRNAs generated from 82%-identical cDNA sequences exhibited target-specific silencing, demonstrating the high degree of specificity for esiRNA[25]. Similar advantages can be expected from a pool of different shRNAs targeting the same transcript in a cell.

Enzymatically prepared shRNA versus siRNA libraries

A major distinction between siRNA and shRNA libraries is that siRNAs directly trigger the RNAi mechanism once introduced into the cytoplasm of the target cells, whereas shRNA is typically expressed in the target cells to trigger RNAi. This fact has consequences as to which system is better suited for specific experiments. Vectors expressing shRNA are the method of choice for persistent knockdown studies. In particular, genomic integration of expression vectors permits phenotypic analyses of a stable depletion of transcripts[4–6].

In contrast, (e)siRNAs have advantages when an acute effect of the knockdown is sufficient and/or desired. Of course, shRNA expression plasmids can also be transfected into cells transiently, and the effect of the knockdown can be monitored. The transfection of plasmid DNA, however, is less efficient and typically more toxic than delivery methods for siRNAs because siRNAs have to be transferred into the cytoplasm only, whereas the expression plasmids have to enter the nucleus.

But many cells, in particular primary cells, are difficult to transfect in general, and this also affects transfections with (e)siRNAs. shRNAs offer the advantage that they can be delivered via viral vectors, which have proven to mediate efficient transfer of the silencing trigger into cells with inefficient transfection rates. However, a major challenge for gene-by-gene screens with viral shRNA libraries is to normalize each well to the same virus titer[42,43]. Without solving this problem, the differences in virus titers will lead to variability in the knockdown level and hence will make analyses of screening data difficult. Also, in contrast to (e)siRNAs, the concentration of intracellularly expressed RNAi triggers is difficult to control. This is especially critical in light of off-target effects (cross-silencing, interferon response) in

Table 1 | Comparison of synthetic and enzymatically prepared RNAi libraries

	Endoribonuclease-prepared siRNA	Enzymatically prepared shRNA (EPRIL; REGS; SPEED)	Chemically synthesized siRNA	Synthetic shRNA
Library preparation	Digestion of dsRNA	Digestion of dsDNA	RNA synthesis	DNA synthesis of the vector insert
	Five main steps of preparing the library	Nine main steps of preparing the library	NA	NA
Key features	Synthesis steps performed *in vitro*	Generation through cloning in *E. coli*	NA	Generation through cloning in *E. coli*
	Limited resource, but easily renewable	Unlimited resource	Limited resource	Unlimited resource
	Inducible silencing not possible	Inducible expression systems available	Inducible silencing not possible	Inducible expression systems available
	Immediate silencing response	Slower silencing response (*in vivo* transcription and Dicer processing necessary)	Immediate silencing response	Slower silencing response (*in vivo* transcription and Dicer processing necessary)
Delivery of silencing triggers	Direct delivery into cytoplasm	Intracellular expression	Direct delivery into cytoplasm	Intracellular expression
	Transient gene silencing	Transient and stable suppression	Transient gene silencing	Transient and stable suppression
	Tighter control of intracellular level of silencing trigger	Intracellular level of silencing trigger varies	Tighter control of intracellular level of silencing trigger	Intracellular level of silencing trigger varies
	Lipofection, electroporation	Lipofection, electroporation, virus-based delivery	Lipofection, electroporation	Lipofection, electroporation, virus-based delivery
Specificity	Low off-target rate through pools of siRNA	Low off target rate when pools of shRNA targeting the same gene	High off-target rate for single siRNA	High off-target rate for single shRNA
Screening mode	Favorable for gene by gene screens	Favorable for selection-based screens (barcoding possible)	Favorable for gene by gene screens	Favorable for selection-based screens (barcoding possible)

NA, not applicable.

RNAi experiments. Because off-target effects can be concentration-dependent, the close control of the silencer concentration delivered to the cells is critical to avoid or reduce unspecific side effects.

In summary, enzymatically prepared siRNA libraries appear to be advantageous for gene-by-gene driven screens, whereas enzymatically-prepared shRNA libraries are superior for studies requiring long-term knockdown and selection-based screens (see **Table 1**).

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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