

RNA interference by mixtures of siRNAs prepared using custom oligonucleotide arrays

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Received November 28, 2004; Revised March 10, 2005; Accepted May 18, 2005

ABSTRACT

RNA interference (RNAi) is a process in which double-strand RNA (dsRNA) directs the specific degradation of a corresponding target mRNA. The mediators of this process are small dsRNAs, of ~21 bp in length, called small interfering RNAs (siRNAs). siRNAs, which can be prepared *in vitro* in a number of ways and then transfected into cells, can direct the degradation of corresponding mRNAs inside these cells. Hence, siRNAs represent a powerful tool for studying gene functions, as well as having the potential of being highly specific pharmaceutical agents. Some limitations in using this technology exist because the preparation of siRNA *in vitro* and screening for siRNAs efficient in RNAi can be expensive and time-consuming processes. Here, we demonstrate that custom oligonucleotide arrays can be efficiently used for the preparation of defined mixtures of siRNAs for the silencing of exogenous and endogenous genes. The method is fast, inexpensive, does not require siRNA optimization and has a number of advantages over methods utilizing enzymatic preparation of siRNAs by digestion of longer dsRNAs, as well as methods based on chemical synthesis of individual siRNAs or their DNA templates.

INTRODUCTION

The phenomenon of RNA interference (RNAi) (1), demonstrated in a plethora of organisms and cell types (1–8), has been extensively used for the specific silencing of different genes. Small interfering RNAs (siRNAs) are mediators of this process (9–14) and can be prepared *in vitro* in a number of ways to be used for RNAi.

Individual siRNAs can be chemically synthesized (11) or transcribed from short DNA templates containing an RNA polymerase promoter (15). Chemical synthesis of RNA is still a very expensive procedure. The efficiencies of different siRNAs for RNAi may differ significantly, and at present there is no way to predict their individual relative potentials. Screening for the most efficient siRNAs using cell cultures is a laborious and expensive procedure. In addition, mutations in a gene target site may affect the efficacy of RNAi (16–18). Therefore, several methods based on the use of siRNA mixtures, which may contain a particular efficient siRNA (or several), have been developed. These include the preparation of siRNA mixtures using RNase III (19) or Dicer (20) enzymes to digest longer double-strand RNAs (dsRNAs). The short RNAs produced as a result of these digestions have been found to be efficient in RNAi.

The disadvantage of these digestive approaches, however, is in their potential for non-specific effects. If a randomly chopped long dsRNA contains areas of homology to other genes, or splice variants of the same gene, the expression of these genes might also be inhibited by cognate siRNAs. In addition, obtaining long dsRNAs also adds to the total cost of, and time required for, these procedures.

It would be more advantageous to use defined mixtures of siRNAs, complex enough to inhibit the desired gene with high probability, but devoid of any siRNAs with unwanted homologies. Besides creating higher target specificity, the use of such mixtures would also alleviate the need to screen for individually active compounds. These mixtures can be created by synthesizing and mixing several siRNAs, prepared by RNA transcription from corresponding DNA oligonucleotide templates. The potential utility of this approach could be limited by the price of these mixtures, which, if prepared by conventional oligonucleotide synthesis, would be proportional to the number of DNA templates required.

Here, we describe a simple, fast and inexpensive method for the preparation of soluble DNA oligonucleotide mixtures using custom oligonucleotide arrays. Arrays of a thousand dif-

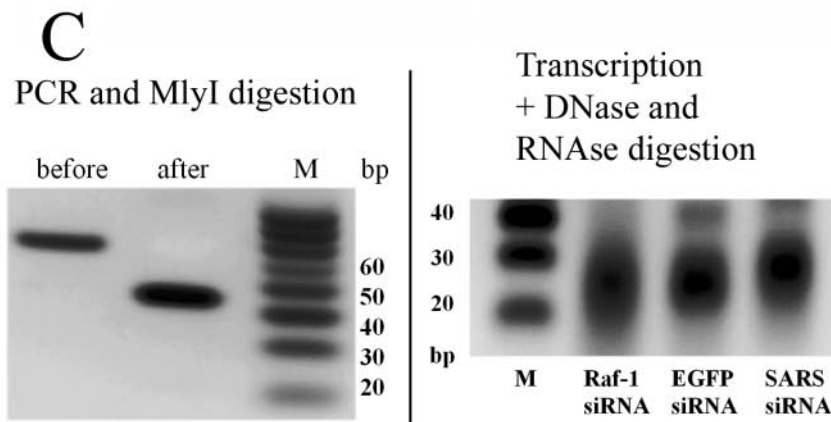
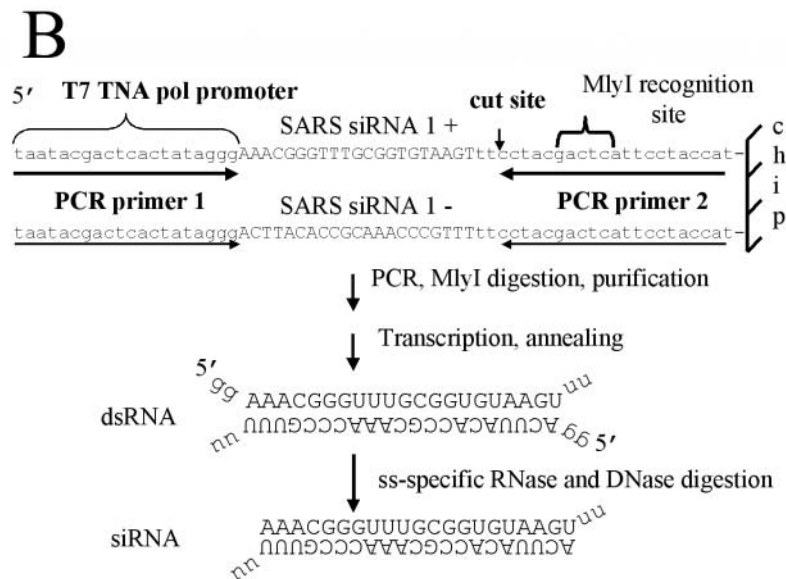
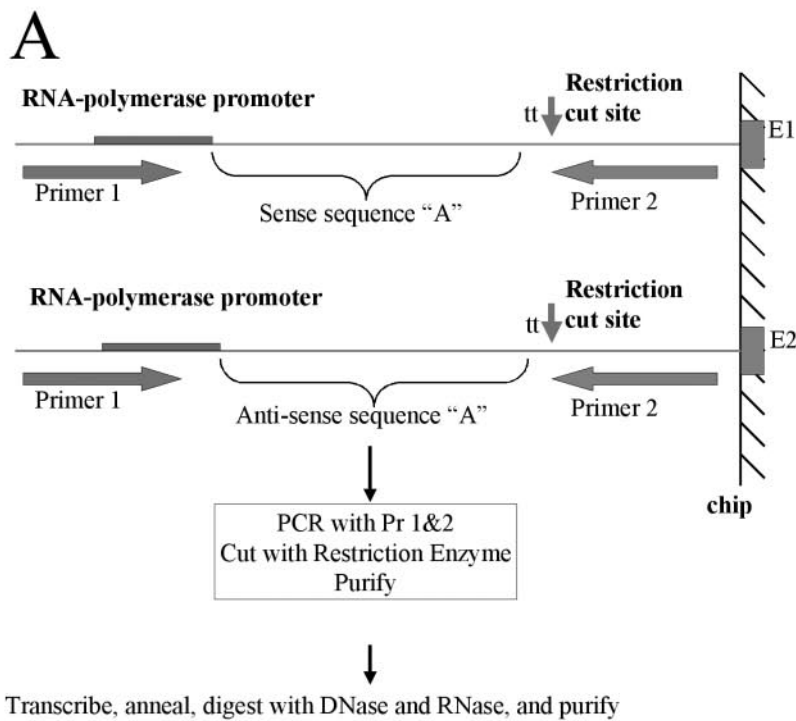
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ferent oligonucleotides can be prepared in 24–48 h using electrochemical synthesis on the surface of a single semiconductor microchip (21). Here, we demonstrate that siRNA pools, prepared using these array-derived DNA oligonucleotide mixtures, is an inexpensive and efficient tool for the inhibition of exogenous as well as endogenous genes in cell cultures.

MATERIALS AND METHODS

Preparation of oligonucleotide arrays

Oligonucleotide arrays were synthesized in the membrane on CombiMatrix semiconductor microchips (CombiMatrix Corp., Mukilteo, WA) as described previously (21). All arrays contained 60 different oligonucleotides, each containing (from 5' end) 20mer T7 RNA polymerase promoter, 21 bases of the corresponding gene (+ or – chain) followed by a TT dinucleotide and 3'-flanking sequence, and attached to the chip membrane at its 3' end (Figure 1). 3'-Flanking sequences were as follows: for control SARS siRNA array 5'-CCTACGACT-CATTCCTACCAT-3', for enhanced green fluorescent protein (EGFP) siRNA array 5'-GAGACGACTCCTAATCGAAT-3' and for Raf-1 siRNA array 5'-CGTAAGACTCAGCGTGA-TGGT-3'. Each flanking sequence contains a recognition site for MlyI restriction endonuclease with its cut site located at the 5' end of the flanking sequence. Underlined flanking sequences are complementary to the corresponding PCR primers used (Primer 2 in Figure 1A and B).

Preparation of the mixtures of soluble siRNA using microarrays

For PCR, semiconductor chips were broken into several pieces, and chip oligonucleotides were amplified in 50 µl mixtures using two primers. One primer was an oligonucleotide resembling T7 RNA polymerase promoter 5'-TAATACGACTCAC-TATAGGG-3', the other primer was complementary to the flanking sequence, as described above. Thirty cycles of PCR were as follows: 95°C for 30 s, 56°C for 30 s, 72°C for 45 s and one cycle at 72°C for 7 min. After PCR, reaction mixtures, without purification, were digested with MlyI for at least 2 h at 37°C. After digestion, soluble double-strand oligonucleotides produced from different pieces of the chip were combined and purified by Qiagen gel-purification kit (without gel electrophoresis) into 100 µl of elution buffer. Transcription and annealing was performed using 24 µl of mixed oligonucleotide templates in 80 µl of reaction volume using the Silencer™

siRNA construction kit (Ambion, Austin, TX). After an overnight incubation at 37°C, dsRNA samples were treated with DNase and single-strand-specific RNase (from the same kit), and purified, using filter cartridges included in the kit, into 100 µl of nuclease-free water. Concentration was measured by absorbance at 260 nm, and yields were typically between 60 and 90 µM.

Cloning and sequencing of DNA oligonucleotides prepared by PCR from on-chip synthesized oligonucleotides

Double-strand DNA (dsDNA) oligonucleotides, amplified by PCR as described above, were cloned into the pCR4-TOPO*TA vector (Invitrogen). DNA was purified using Wizard plasmid purification kit (Promega) and sequenced at Davis Sequencing (Davis, CA).

Gel electrophoresis of DNA and siRNA

Samples were separated in precast 4% agarose gels (containing ethidium bromide) in TBE buffer (Cambrex BioScience Rockland, Inc., Rockland, ME). A total of 10 bp DNA Step Ladder from Promega was used in these experiments as molecular weight marker.

Cell culture and transient transfections

HeLa cells were grown in EME medium (ATCC) supplemented with 10% fetal calf serum, 1 mM L-glutamine and 100 U/ml of penicillin–streptomycin at 37°C, with 5% CO₂. Cells were seeded in six-well tissue culture plates and grown to 50–70% confluency for transient transfection experiments. For assessing the effect of the EGFP siRNA pool on exogenous EGFP expression, a series of concentrations (0, 12.5, 25 and 50 nM) of EGFP siRNA or control SARS siRNA pools were each co-transfected with 0.7 µg/well of the pEGFP-N1 expression vector (Clontech) and 0.5 µg/well of pSEAP reporter vector (Clontech), which was used as a control for transfection efficiency. For examining endogenous c-raf gene silencing, a similar series of concentrations of human Raf-1 siRNAs or control SARS siRNA pools were each co-transfected with 0.7 µg/well of the pEGFP-N1 vector (Clontech), which was used as a control for transfection efficiency. Transfection experiments were performed using LipofectAMINE 200 Reagent (Invitrogen) according to the manufacturer's instructions. The functional effects on expression of target genes were measured by fluorescence microscopy using fluorescein

Figure 1. (A) General scheme for the preparation of siRNA mixtures using custom oligonucleotide arrays. E1 and E2 are electrodes, over which different oligonucleotides 1 and 2 are synthesized. Three sets of siRNA were designed. A control set was composed using information on SARS coronavirus CUHK-W1 (accession no. AY278554) and contained 30 different double-strand siRNAs cognate to viral mRNA coding for RNA-dependent RNA polymerase and metal-binding NTPase/helicase domains. To inhibit the expression of exogenous GFP, we designed 30 siRNAs covering the full sequence of its mRNA, starting from the first ATG codon (pEGFP-N1, accession no. U55762) and shifted relative to each other by 21 bases. To inhibit the expression of the endogenous Raf-1 proto-oncogene, we designed 30 siRNAs derived from the entire sequence of ~3000 bases of the human Raf-1 mRNA (accession no. X03484) (22). The first siRNA started at base –129 relative to the initiator ATG codon. Each 21mer oligonucleotide was separated from the next by 90 bases. All oligonucleotide sequences (sense chain) are included in the Supplementary Material. (B) Detailed scheme for the preparation of SARS siRNA mixture showing oligonucleotide sequences used for promoter and flanking regions, and a pair of internal complementary chains encoding SARS siRNA #1 as an example. Each DNA oligonucleotide contained a T7 RNA polymerase promoter preceding the specific siRNA templates. (C) DNA and RNA products generated during siRNA mixture preparation. Left: 4% agarose gel analysis of soluble dsDNA oligonucleotide siRNA templates prepared by PCR amplification of array oligonucleotides as shown in (A and B). PCR was performed using two primers, common for all the oligonucleotides synthesized on a particular chip. To exclude the possibility of cross-contamination between siRNA pools during amplification, different pairs of primers were used for chips designed with SARS, GFP and Raf-1 oligonucleotides. One primer, containing a T7 RNA polymerase promoter, was common for all sequences; the other, PCR primer 2, was different in each case (see Materials and methods). Before: PCR products before digestion with MlyI (63 bp); after: PCR products after digestion with MlyI and purification (43 bp). M: DNA oligonucleotide markers. Right: siRNA mixture preparations analyzed by 4% agarose gel electrophoresis. The mixtures of siRNAs migrate at an approximate size of 25 bp dsDNA oligonucleotides.

isothiocyanate filter set (EGFP) and by western blotting (Raf-1) after 24 h (EGFP and Raf-1) and 48 h (EGFP) post-transfection. In each case, at least three independent transfection experiments were performed.

Western blotting of EGFP, beta-actin and Raf-1

The expression of EGFP, beta-actin and Raf-1 proteins were examined by western blotting. Briefly, siRNA-treated cells were lysed in cold passive lysis buffer (Promega) for 30 min on ice. Protein concentrations in samples were measured using Bio-Rad Protein Assay (Bio-Rad). Equal amounts of proteins (usually 2 μ g) from each sample were mixed with 2 \times SDS buffer (Bio-Rad), heated at 95°C for 5 min and fractionated by 4–15% gradient SDS-PAGE. Gels were then transferred onto Immobilon-P polyvinylene difluoride membrane (Bio-Rad) overnight at 4°C by wet transfer system (Bio-Rad). Membranes were blocked in TBST (50 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.05% Tween-20) containing 10% non-fat milk, and then probed with rabbit anti-Raf-1 antibody (1:500; Sigma), rabbit anti-EGFP antibody (1:500; Clontech) or mouse anti-beta-actin antibody (1:500; Sigma) in TBST containing 1% non-fat milk for 2 h at room temperature. Western blots to detect beta-actin and EGFP were performed by re-probing membranes used for Raf-1 detection. After washing three times in TBST, membranes were further incubated with the appropriate anti-rabbit or anti-mouse secondary antibodies (both from Jackson ImmunoResearch Laboratories) conjugated to horseradish peroxidase (1:1000) for 1 h at room temperature. Membranes were washed three times in TBST, two times in TBS, and developed using LumiGLO chemiluminescent substrate kit (KPL, Gaithersburg, MD). Images were recorded, and band intensities on western blots were quantified, using the CCD-based FluorChem™ 8000 Imaging System (Alpha Innotech Corp.).

RESULTS AND DISCUSSION

Figure 1A and B shows our scheme for the preparation of a mixture of siRNAs using a custom DNA oligonucleotide array. Using this scheme, we prepared three sets of siRNA mixtures, each containing 30 double-strand siRNAs: negative control SARS siRNAs, EGFP siRNAs and Raf-1 siRNAs. We presumed that virally targeted SARS siRNAs would not affect the expression of the genes, which we used to assess specific RNAi effects, EGFP and Raf-1, and that they could be used as negative controls in our experiments. The siRNAs designed all had a similar structure: 21 bp corresponding to the gene sequence and two unpaired U's at each 3' end (Figure 1B).

Figure 1C shows agarose gel electrophoresis of oligonucleotides and dsRNA samples from different steps of the siRNA mixture preparation. After PCR, the resulting dsDNA oligonucleotides were treated with MlyI restriction enzyme to remove one flanking sequence (on the opposite side from T7 promoter) in order to create precise templates for transcription (Figure 1B). Agarose gel electrophoresis (Figure 1C) confirmed both the correct size and complete digestion of PCR fragments. To confirm the diversity and the quality of chip-derived DNA templates, PCR products were also cloned (before digestion with MlyI enzyme) for sequencing. The 18 clones examined represented 16 different siRNA-coding

sequences, and each siRNA-coding 21mer contained 100% correct sequence.

Transcription of the original mixture of templates produced corresponding single-strand RNAs (ssRNAs) containing a GG-pair at their 5' ends (as a result of T7 polymerase transcription initiation), 21 bases cognate to the selected gene, and a UU-pair at their 3' ends. All complementary chains were synthesized together in a single reaction, and dsRNAs were formed directly during the transcription process. No additional melting–annealing steps were performed because the long incubation at 37°C is sufficient for the formation of dsRNAs. Reactions were treated with DNase to remove the DNA oligonucleotide templates, and with 5' single-strand-specific RNase to remove the GG-overhanging bases from the 5' ends of dsRNAs. After purification, the typical final concentrations of the siRNA mixtures, prepared from a single microchip, were ~60–90 μ M (or 80–120 μ g total siRNA in 100 μ l), which is sufficient for many cell culture experiments. It is clear that the electrophoretic mobility of these dsRNA mixtures is between the 20 and 30 bp dsDNA markers (median at 25 bp), which corresponds perfectly to the expected mobility of 21mer dsRNAs with two overhanging nucleotides at each 3' end (Figure 1C).

The functional activity of siRNAs prepared using custom oligonucleotide arrays was tested in RNAi cell culture experiments. Results of an RNAi experiment with exogenous GFP transfected into HeLa cells together with siRNA preparations (control SARS or EGFP siRNA) are shown in Figure 2. Transfection efficiency was similar in different samples, based on the activity of the co-transfected marker, secreted alkaline phosphatase (SEAP) (data not shown). It is clear that the expression of EGFP is significantly reduced by treatment with the GFP siRNA mixture but not by the control SARS siRNA mixture. The inhibitory effect was clearly visible 24 h

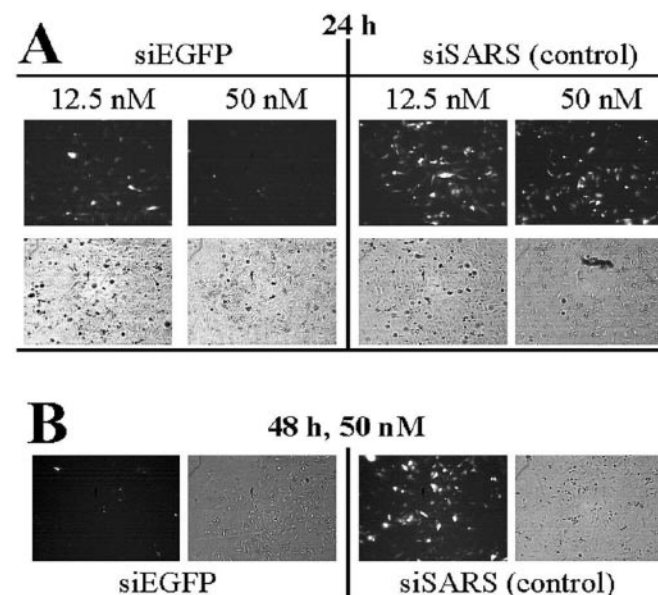


Figure 2. Inhibition of EGFP expression in HeLa cells by RNAi analyzed by fluorescence microscopy. The same microscope field is shown in white light (all cells are visible) and fluorescence light (only cells expressing EGFP are visible) for each sample. (A) Twenty-four hours post-transfection; (B) 48 h post-transfection.

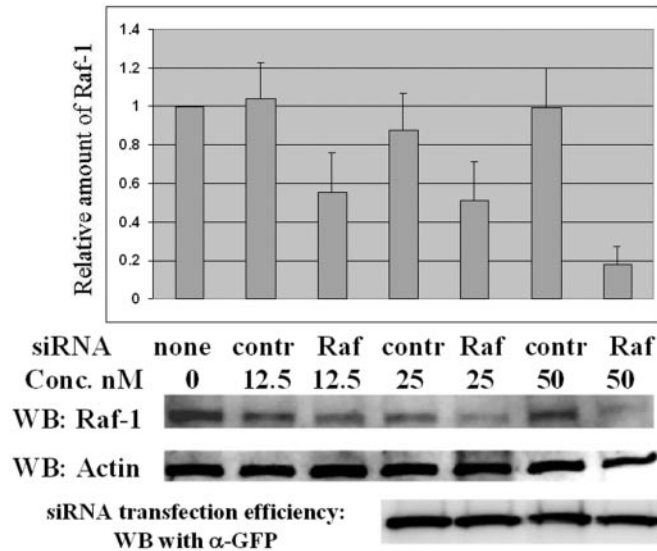


Figure 3. Inhibition of endogenous Raf-1 expression in HeLa cells by RNAi analyzed by western blotting. Top: light intensity for each Raf-1 band was normalized to the beta-actin band in the same lane and, for comparison between different experiments (4 total), these data were normalized relative to the untreated controls (without siRNA). The differences between Raf-1 siRNA-treated cells and control SARS siRNA-treated cells were statistically significant at each concentration examined ($P < 0.006$ by paired *t*-test). Bottom: typical western blotting (WB) data from a single RNAi experiment. Chemiluminescence images are shown in negative. None, control without siRNA; contr, control SARS siRNA.

after transfection at 12.5 nM of siRNAs (total concentration) and was almost complete at 50 nM (Figure 2).

Figure 3 demonstrates that our siRNA mixtures can significantly reduce the expression of endogenous Raf-1 genes. HeLa cells were transfected with none, control (SARS) or Raf-1 siRNA mixtures at different concentrations. pEGFP reporter plasmid was included in the transfection mixtures as a control for transfection efficiency. At the bottom of the figure is shown western blotting data from a typical RNAi experiment (chemiluminescence images are reversed). The graph in this figure demonstrates the average inhibitory effect in four independent cell-culture experiments. The effect of Raf-1 siRNA on the level of Raf-1 protein is dose-dependent; at a 50 nM siRNA concentration, Raf-1 expression is inhibited by $\sim 80\%$ compared with the 50 nM siRNA control. Since this level of inhibition is comparable with the overall transfection efficiency ($\sim 80\%$, calculated by counting EGFP-positive cells), it appears that Raf-1 expression is almost completely inhibited in the population of successfully transfected cells at this concentration. It is evident from western blot control data and from a visual inspection of treated cells that siRNA mixtures were not toxic at the concentrations used during our experiments.

The results presented clearly demonstrate that our method of preparing siRNA using custom DNA oligonucleotide arrays is efficient in the production of functionally active compounds. The inhibitory effects of the siRNA mixtures on gene expression in this study were comparable with, or better than, those of mixtures prepared by RNase III digestion of full-length EGFP dsRNA (data not shown). In addition to the dimeric dsRNA described here, siRNAs can also be designed as

hairpin loops (3). This would double the potential number of different siRNAs that can be synthesized per chip, as both strands of the RNA duplex are encoded within a single oligonucleotide. Hairpin loops might also be more favorable for very complex mixtures of siRNAs owing to their high potential for intra-molecular double-strand formation. The custom nature of the CombiMatrix array platform provides for the incorporation of various elements of structural and informatic design in generating defined mixtures of siRNAs. For example, in our design of the control SARS siRNAs, we chose only gene fragments that contain minimal homology to published human genes in hope of minimizing non-specific effects on cells. Hence, any potentially cross-reactive sequences (e.g. sequences that contain homology to closely related known targets or, perhaps, those having minimal homology to a particular 'background' genome) can be excluded from a set of siRNAs in order to optimize them for gene-specific targeting.

Different siRNA mixtures can be prepared from a single microarray in a variety of ways, e.g. by physical breaking of the array into individual pieces, by the use of different primers for the amplification of different sets of siRNA template DNA oligonucleotides, or, in addition, by the use of different RNA polymerase promoter sequences introduced into siRNA template DNA oligonucleotides. The more different siRNAs, or the more different siRNA pools, that are prepared using the same microchip, the lower the price of each siRNA, or pool, would be. Preparation a single pool of siRNA consisting of 30 different double-strand siRNAs by conventional transcription methods requires sixty 29mer DNA oligonucleotides (e.g. using Ambion's Silencer™ siRNA construction kit). Assuming a lowest possible price of \$0.14 per base for oligonucleotides prepared in-house, the minimal total cost for these oligos alone would be about \$250. This is about twice the price of preparing a single oligonucleotide array on the current CombiMatrix platform, including all expenses, irrespective of the number of different oligonucleotides made (up to 1000). Therefore, CombiMatrix oligonucleotide arrays are cheaper than conventional methods of preparing DNA templates for siRNA transcription; depending on the number of oligonucleotides in the mixture, or the number of mixtures, they can be significantly cheaper. Because semiconductor devices are scalable, without price increase, the price advantage over individually synthesized and mixed DNA oligonucleotide templates will increase further with the use of arrays with higher densities of different oligonucleotides.

Although the actual data are incongruous, two types of off-target effects have been described in the literature: (i) a non-specific effect, which is independent of sequence homology with a particular siRNA and (ii) a specific effect, when non-targeted genes with partial complementarity to a particular siRNA directed against the target gene are downregulated (23–25). These effects can be problematic, and are complex and not readily reproducible, because they depend upon the particular system and target proteins that are used. In the present study, we did not investigate whether our siRNA mixtures would reduce potential off-target effects. This was outside the scope of our present work as it represents a more general issue concerning the use of RNAi technology. Nevertheless, a simple assumption suggests that by excluding

the known complementary or homologous sequences one may prevent at least the 'specific type' of off-target effect. Although this still may not remove possible non-target effects owing to residual homologies that are potentially impossible to avoid completely due to the enormous size and undefined nature of some genomes, the use of complex mixtures of defined siRNA molecules may also offer a unique advantage over current methods. Because each efficient siRNA has an optimum concentration when its effect is maximal (the practical effective concentration range is 10–100 nM), if such an siRNA molecule, homologous to an off-target gene, were present in a defined mixture, it is likely that its concentration would be below the effective range. Thus, since a complex mixture may include 30–50 different siRNAs at a total concentration of 10–100 nM, this may, theoretically, lessen the off-target effect of that particular siRNA molecule because it is contained within a mixture. Whether siRNA mixtures can reduce either type of off-target effect or not will, hence, be the focus of future research.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

ACKNOWLEDGEMENTS

The authors thank Marcello Caraballo and Matt Harvey for the synthesis of oligonucleotide arrays, and Kevin Schwarzkopf and Irina Oleinikova for technical help. The Open Access publication charges will be paid by the authors.

Conflict of interest statement. None declared.

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