

Multiplexing siRNAs to compress RNAi-based screen size in human cells

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ABSTRACT

Here we describe a novel strategy using multiplexes of synthetic small interfering RNAs (siRNAs) corresponding to multiple gene targets in order to compress RNA interference (RNAi) screen size. Before investigating the practical use of this strategy, we first characterized the gene-specific RNAi induced by a large subset (258 siRNAs, 129 genes) of the entire siRNA library used in this study (~800 siRNAs, ~400 genes). We next demonstrated that multiplexed siRNAs could silence at least six genes to the same degree as when the genes were targeted individually. The entire library was then used in a screen in which randomly multiplexed siRNAs were assayed for their affect on cell viability. Using this strategy, several gene targets that influenced the viability of a breast cancer cell line were identified. This study suggests that the screening of randomly multiplexed siRNAs may provide an important avenue towards the identification of candidate gene targets for downstream functional analyses and may also be useful for the rapid identification of positive controls for use in novel assay systems. This approach is likely to be especially applicable where assay costs or platform limitations are prohibitive.

INTRODUCTION

Technologies that exploit the endogenous RNA-based gene silencing mechanism, RNA interference (RNAi), have developed rapidly for the dissection of gene-function relationships and as a means of furthering molecular target analysis (1,2). The value of large, high-throughput RNAi screens in human cells has recently been demonstrated (3–15). However, a number of practical limitations

are associated with large RNAi screens [using either small interfering RNAs (siRNAs) or expressed short hairpin RNAs, termed shRNAs]. Those limitations include the need for extensive automated liquid handling and/or the requirement for large amounts of transfection and assay reagents, which can make such approaches inaccessible to many laboratories. In addition, some assay platforms are incompatible with large-scale analysis because of the limited number of samples that can be assayed at any one time. Furthermore, the number of 'hits' in larger unbiased screens may only correspond to a small fraction of the total targets evaluated, retrospectively making the majority of such screens superfluous. To address some of these issues, strategies using pools of shRNAs labeled with unique 'bar codes' have been described (16,17). Bar codes allow for the identification of enriched or depleted shRNAs among treated cell populations. Unfortunately, such a method is not applicable to synthetic siRNA-based screens. Thus, we investigated a strategy designed to reduce the size of synthetic siRNA-based screens. This approach involves the use of multiplexed siRNAs directed against multiple gene targets. Since the activity of such multiplexes has not been studied extensively at the molecular level, we began by characterizing the activity of co-administered siRNAs. We found that multiplexed siRNAs corresponding to at least six gene targets yielded reductions in associated mRNA levels comparable to those induced by individual siRNAs. We next examined the usefulness of a multiplex-based strategy in a screen for targets that negatively affect the growth of a highly tumorigenic cell line. We discovered that members of a library comprising randomly multiplexed siRNAs exhibited highly reproducible phenotypic effects and permitted the rapid identification of candidate targets that affected cell viability. Additionally, by screening a second library, constructed such that no two constituents of a multiplex in the first library were co-members in the second, we were able to identify active multiplex members without extensive deconvolution. We offer that such a strategy

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can be a good starting point for the rapid identification of candidate targets, especially where assay costs or low-throughput equipments are limiting.

MATERIALS AND METHODS

Cell lines

HCT-116 colon cancer cells and MDA-MB-231 breast cancer cells (both members of the NCI-60 cell panel) were obtained from the NCI Developmental Therapeutics Program (DTP) (<http://dtp.nci.nih.gov/>) and were maintained in RPMI containing 5% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO₂. The cells were passaged every four to five days. The choice of cell line for characterizing RNAi directed against individual target genes was determined by pre-assessing a gene's mRNA level (assayed using a gene-specific branched-DNA/RNA assay; see below) to ensure sufficient levels for accurate measurement. Three additional cell lines (NCI/ADR-RES, MCF10A and MCF7) were used to analyze specific gene targets, one gene at the mRNA level and two genes at the protein level (see Supplementary Data for additional details).

Transfection of synthetic siRNAs

The synthetic siRNAs used in these studies were designed and synthesized by Qiagen Inc., Germantown, MD, USA. Target sequences are listed in Supplementary Table 1. For characterization studies, each siRNA (2.5 or 5 pmol) was added to individual wells in a 96-well plate in 25 µl of serum-free RPMI and complexed with siLentfect transfection reagent (Bio-Rad, Hercules, CA, USA) in 25 µl of serum-free RPMI using a final lipid:siRNA ratio of 2:1 (w/w). The resulting mixture was allowed to complex for 30 min at ambient temperature. All liquid-handling steps were automated (Qiagen Robot 8000, Qiagen, Germantown, MD, USA). Next, cells (5000) were added in 50 µl RPMI supplemented with 10% FBS to yield transfection mixtures consisting of 25 or 50 nM siRNA in RPMI with 5% FBS. The final mixture was incubated at ambient temperature for 45 min before being placed at 37°C in a humidified atmosphere containing 5% CO₂. Cells were harvested 48 h post-transfection for mRNA analysis. Each siRNA was evaluated in quadruplicate at both 25 and 50 nM by comparing target mRNA levels to those found in cells transfected with either 25 or 50 nM negative-control siRNA. For protein studies, transfections were performed as described above except that they were conducted in 6-well plates and all reagent amounts were scaled up 30-fold. Protein studies were conducted using 50 nM siRNA. As with evaluation at the mRNA level, protein levels were compared with those found in cells transfected with negative-control siRNA.

Multiplexes of siRNAs were transfected in the same way as described for mRNA characterization studies except that they were used at a final concentration of 60 or 120 nM (10 nM of each individual siRNA) and 0.7 µl of Oligofectamine (Invitrogen, Gaithersburg, MD, USA)

was added per well as the transfection reagent. After complex formation, 5000 MDA-MB-231 cells were added. Analogous protocols were used for transfection of MCF10A cells except that MCF10A growth medium was used (see Supplementary Data). Multiplex experiments were incubated for two days for mRNA analysis and four days for cell viability analysis. Multiplexed-siRNA screens were conducted in the interior 60 wells of 96-well plates. For cell viability, all multiplexes were assayed in duplicate. Cell viability was assayed as described below, and plate median values were used for normalization to compare samples assayed on different plates. The arrayed screen was conducted as described for the multiplex screens. For the arrayed screen, both siRNAs targeting a given gene were evaluated as a pair, each used at 10 nM. For deconvolution and target validation, the two siRNAs targeting a given gene were evaluated as a pair, each used at 10 nM, and compared with cells transfected with negative control siRNA (20 nM). For evaluation of non-specific effects, the two siRNAs targeting a given gene were evaluated as individuals (20 nM) and compared with cells transfected with negative-control siRNA (20 nM).

RNA, DNA sequence, protein and cell viability assays

For characterization of RNAi, gene-specific transcript levels were measured using a branched DNA-based assay (18) (QuantiGene Reagent System, Panomics, Fremont, CA, USA). Briefly, cells were lysed 48 h after siRNA transfection and 5–80 µl of lysate was assayed, depending on transcript abundance. Gene-specific mRNA levels were normalized to human cyclophilin B (*PPIB*) mRNA levels (probe set nts 74–432). For transcript splice variant analysis, RNA was extracted with Trizol and cDNA synthesized at 42°C using oligo-dT/random hexamers provided by the ThermoScript RT-PCR system (Invitrogen). Samples for i-Cycler-based (Bio-RAD) real-time quantitative PCR (qRT-PCR) using SYBR green as the fluorochrome and 25 µl reactions were run at 95°C denaturing, 50–60°C re-annealing (depending on the requirements of the specific transcript), and 72°C extension for 20–40 cycles (depending on the requirements of the specific transcript). Genomic DNA was purified using the Puregene DNA purification system (Gentra System, Minneapolis, MN, USA), and sequence analysis of DNA or cDNA PCR products primers was run using primers flanking the sequence of interest. PCR products were size separated by agarose gel electrophoresis and purified by extraction with phenol and ethanol. The sequences of DNA primers used for PCR amplification and sequencing are shown in Supplementary Table 2. western blot analysis was performed as previously described (19); see Supplementary Table 3 for the antibodies used. Multi-array ECL assay analysis was conducted according to manufacturer's instructions for each protein (MesoScale Discovery, Gaithersburg, MD, USA). The reduction of resazurin to resofurin, read as fluorescence (560Ex/590Em), was used to assay cell viability (Cell Titer Blue Reagent, Promega, Madison, WI, USA).

Statistical analysis

Two-tailed Student's *t*-tests and correlation analysis were done using functions in Excel (Microsoft).

RESULTS

Most synthetic siRNAs mediate RNAi

To interpret RNAi functional data fully, some characterization of the RNAi mediated by any given effector molecule is useful, as the degree to which mRNA and cognate protein levels are affected will influence the observed functional effects. Consequently, we analyzed the RNAi induced by a large subset of our siRNA library. The targets of this library include genes with a broad range of known and putative cancer-related functions including classical oncogenes and tumor suppressors, established and putative anti-cancer targets (primary and metastatic) and proteins associated with anti-cancer chemotherapeutic responses. Overall, we characterized the target specific effects of 258 synthetic siRNAs on mRNA levels corresponding to 129 human genes (two siRNAs per target) (Supplementary Table 4). To compare relative efficacies, we ranked the percentage decrease in target mRNA as compared to levels found in cells transfected with negative-control siRNA (Figure 1a). We found that 64% of siRNAs mediated at least a 50% change in mRNA levels with a median percentage decrease in gene target mRNA levels of 69% and a maximum of 97%. A number of characterized siRNAs (37 in total) were further examined for their affect on target protein levels using western blots and/or multi-array ECL

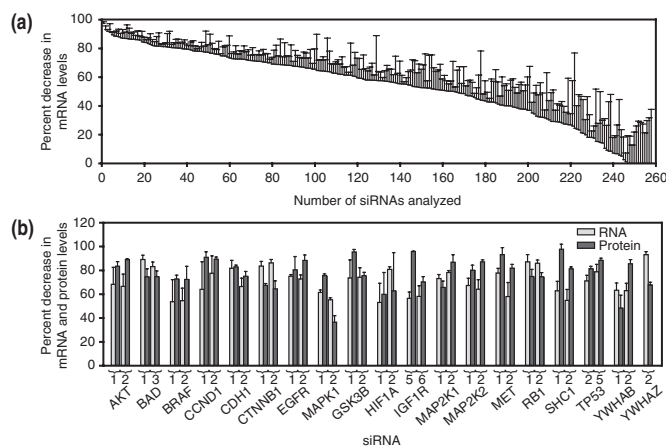


Figure 1. Quantification and analysis of the RNAi mediated by synthetic siRNAs targeting over 100 human genes. **(a)** A ranking of the percentage silencing mediated by 258 siRNAs corresponding to 129 human genes. The percentage silencing was determined by comparing the mRNA levels for each gene following transfection with 50 nM target-specific siRNA to levels found in cells transfected with 50 nM negative-control siRNA. Data is shown as the mean percentage decrease in target mRNA levels observed for four independent parallel transfections (plus SD). Analysis was conducted in HCT-116 (118 siRNAs), MDA-MB-231 (138 siRNAs) and NCI/ADR-RES (two siRNAs). **(b)** The percentage decrease in mRNA and protein levels for 19 genes (two siRNAs per gene). mRNA levels were measured 48 h post-transfection, whereas total protein levels were measured 48–96 h post-transfection.

assays (19 proteins) (Supplementary Figure 1). In ~70% of cases, the measured decrease in protein was greater than or equal to that observed for the corresponding decrease in mRNA levels (Figure 1b). Notably, we identified a number of cases where RNAi was hindered by the presence of SNPs or uncharacterized transcript splice variants (Supplementary Figure 2).

Molecular validation of multiplexed siRNAs

We next examined the feasibility of multiplexing siRNAs in order to reduce the size of traditional screens. Although the use of siRNA pools targeting the same gene is a relatively common practice, only a limited number of studies using combinations of siRNAs for the simultaneous targeting of more than one gene have been reported. For example, co-administration of siRNAs has been used to dissect the roles of Rab proteins in dense-core vesicle exocytosis (20), and co-knockdown of FAS and HER2 has been shown to promote apoptosis synergistically in cancer cells (21). However, in the vast majority of those types of studies, no more than two targets were co-silenced, and the effects of siRNA co-administration were usually characterized only at a phenotypic level. Therefore, we set out to validate conditions for the simultaneous knockdown of multiple targets by RNAi. Experimental multiplexes consisting of six siRNAs directed against three unique gene targets (two siRNAs per target) yielded silencing comparable to that exhibited by individual siRNAs in the breast cancer cell line MDA-MB-231 (Figure 2a). Importantly, the total siRNA concentration was held constant for these experiments such that the sum concentration of all multiplex members equaled that of individually evaluated siRNAs. Similar results were observed when multiplexing twelve siRNAs against six gene targets (Figure 2b). Multiplexes retained activity when using gene targets other than those described in Figure 2 (data not shown) or when conducting studies in other cell lines, including MCF10A (Figure 2c).

Screening siRNA multiplexes

To see if we could use this multi-gene RNAi approach to streamline an siRNA screen in practical terms, we generated a library comprising 135 randomly generated multiplexes, each of which consisted of six siRNAs corresponding to three unique gene targets. We then used this library in a cell viability screen of MDA-MB-231 cells. Multiplexes were evaluated at a total concentration of 60 nM (10 nM of each individual siRNA) and cell viability was assessed 96 h post-transfection. The library was screened in triplicate, twice in parallel and once in a separate experiment using cells from a different passage (Figure 3a). The screens conducted in parallel exhibited good correlation (Figure 3b), sharing 19 of the 20 multiplexes that ranked highest in terms of a decrease in cell viability. The third, independent screen also showed good correlation; 16 of the same 19 multiplexes were found among its top 20. Furthermore, the vast majority of multiplexes showed little activity. For example, ~80% of multiplexes exhibited cell viability within 10% of the

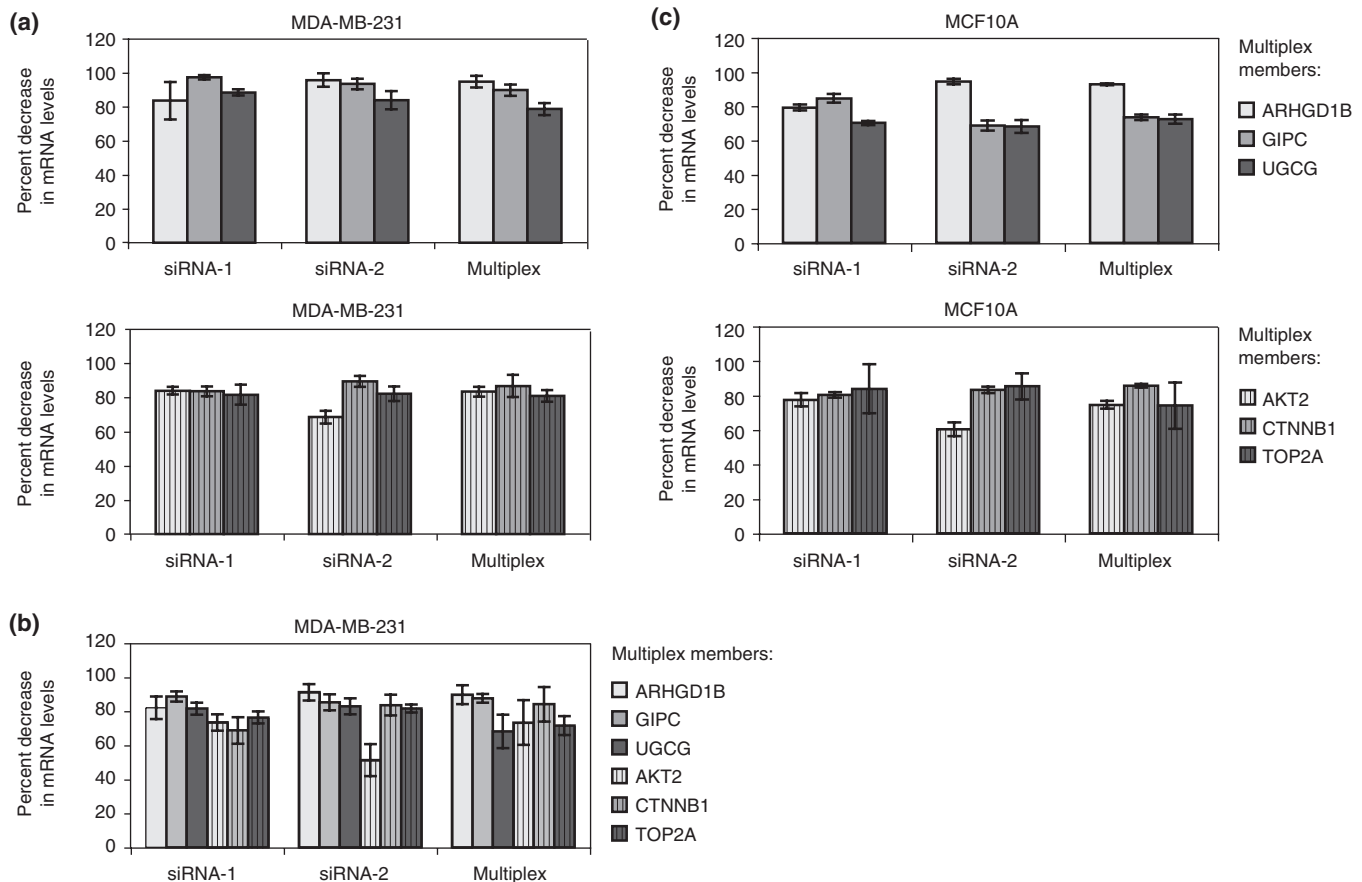


Figure 2. Multiplexed siRNAs retain comparable efficacy to that exhibited by their individual counterparts. (a) Two multiplexes consisting of six siRNAs corresponding to three targets (two siRNAs per target) were evaluated for their ability to reduce cognate mRNA levels as compared to the activity of respective individual siRNAs in MDA-MB-231 cells. Total siRNA concentrations were held constant for all experiments such that individual siRNAs (siRNA-1 and siRNA-2) were tested at 60 nM and multiplexed siRNAs were tested at an individual siRNA concentration of 10 nM (60 nM total). Target-specific mRNA levels were compared to those found in cells transfected with negative-control siRNA. (b) Same as in (a) except that the multiplex consisted of twelve siRNAs corresponding to six targets. In this case, the total siRNA concentration was held constant at 120 nM again using 10 nM of each individual component within the multiplex. (c) Same as in (a) except that the experiments were conducted in MCF10A cells. Similarly, multiplexes retained activity when using gene targets other than those described here or when conducting studies in additional cell lines (data not shown).

median value (Figure 3a). This multi-gene siRNA approach enabled us to streamline a screen of over 800 siRNAs to an extent that required no automated liquid-handling steps. A deconvolution established the top four gene targets responsible for reduced cell viability in the overall screen (Figure 3c, Multiplexes A–D). Deconvolution also identified multiplexes whose activity seemingly had not arisen from the action of a single multiplex constituent, but rather through some cumulative effect of multiple members (Figure 3c, Multiplexes E and F).

The use of constrained multiplexed siRNA libraries

To help identify gene target(s) within any given multiplex that contribute to the assayed phenotype without additional deconvolution, we generated a second random library with the constraint that no two targets contained within a multiplex in the first library were co-members of a multiplex in the second. Evaluation of both libraries in MDA-MB-231 cells permitted easy identification of the key gene targets that markedly decreased cell

viability (Figure 4). Several of the genes identified in the screen are highly relevant targets of anti-cancer therapeutic strategies. Examples are the ribonucleotide reductase enzyme, both subunits of which were identified here (RRM1 and RRM2) (22), the Rho-GTPases (RHOA) (23), polo-like kinase 1 (PLK1) (24), mTOR (FRAP1) (25) and MEK1 (26). Other genes identified have been proposed as targets but, often because of the difficulty in developing inhibitors, have not been studied as fully. An example is MMP2 (27). Although this strategy represents only a modest reduction in the total screen size as compared to screening individual siRNAs in an arrayed format, it was again accomplished without automated liquid handling and may be especially applicable if multiplexes comprising a greater number of constituents (e.g. six gene targets per multiplex) are used.

Comparing multiplex and arrayed screens

To help examine the occurrence of false-negative results in multiplex-based screening, we conducted an arrayed

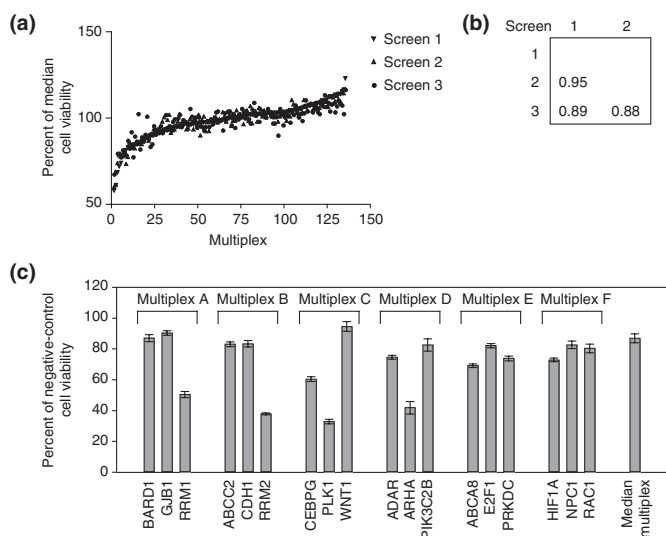


Figure 3. Comparing screen reproducibility and deconvolution. (a) Graphical representation comparing three cell viability screens conducted in MDA-MB-231 cells. The first two screens (1 and 2) were conducted in parallel, whereas the third (3) was conducted under independent conditions. The y-axis reflects percent of the median value. (b) The correlation between screens. (c) Deconvolution of the top twenty multiplexes revealed those containing the top four reducers of cell viability (multiplexes A–D), while also identifying multiplexes whose ranking does not appear to result from the activity of an individual target, but perhaps through an additive effect (multiplexes E and F). All values are reported as the percent viability of cells transfected with a negative-control siRNA. As an additional reference, a multiplex found to approximate the median value in all three screens of library #1 was also used.

screen comprising the same siRNAs used in the multiplex library. For the arrayed screen, both siRNAs targeting a given gene were combined and evaluated at a total concentration of 20 nM (10 nM of each individual siRNA). As with the multiplex screens, cell viability was assessed 96 h post-transfection. We found that 80% of siRNAs representing the most significant down-regulators of cell viability in the arrayed screen, those resulting in cell viability greater than three SD from the median value, were identified using a multiplexed strategy (4 of 5 genes). This comparison helps to demonstrate that a multiplex-based approach can identify a majority of the strongest hits within a given synthetic siRNA library.

DISCUSSION

As the scale of an siRNA-based screen increases, the need for automated liquid handling and large amounts of assay reagents can become limiting for many laboratories. Additionally, large-scale screens may be incompatible with assays using limited capacity equipment, for example ‘high content’ screening approaches using imaged-based phenotypic analysis. In many cases, the scale of a screen may be increased further by the need to evaluate siRNAs under a variety of conditions. For example, screens for targets that affect compound activity should minimally require the evaluation of siRNAs in both the presence and absence of compound and may optimally require

evaluation at multiple doses. Thus, strategies for reducing screen size would be advantageous in a number of contexts.

Multiplexed-based approaches have often been used in small-molecule screens and in studies of protein–protein interactions. Here we extend that strategy in order to reduce the size of siRNA-based screens. We first characterized the activity of multiplexed siRNAs at a molecular level and found that multiplexed siRNAs reduced the mRNA levels of at least six targets simultaneously. This is interesting as others have reported a reduction in siRNA activity as a function of co-transfection with inactive siRNAs (28). Our results suggest that it is possible to achieve optimal transfection conditions, whereby multiplexed siRNAs retain activity comparable to that exhibited by their individual constituents in, at least, some cell lines. By validating the ability of multiplexed siRNAs to co-regulate several genes, we suggest that siRNA-mediated RNAi could be used more extensively, than currently reported, for the study of interactions within biological pathways and networks. This type of analysis could be especially valuable for the development of anti-cancer drugs, as it could assist in identifying molecular target combinations that generate particularly lethal phenotypes.

As with all RNAi-based studies, off-target effects may result in false positives. This study uses multiplexes of siRNAs to streamline screens. The use of multiplexes could result in a higher occurrence of off-target effects. To potentially reduce these effects, we used individual siRNAs within multiplexes at relatively low, but effective, concentrations (10 nM). Some studies have demonstrated that using siRNAs at low concentrations can reduce off-target effects. For example, lowering siRNA concentrations from 100 to 20 nM eliminated the off-target effects associated with 15 different siRNAs corresponding to three different genes (29). Conversely, other studies have indicated that off-target effects do not simply result from high siRNA concentrations and cannot be eliminated by using less siRNA (30,31). Although those studies have shown that off-target effects exhibit dose responses that mirror on-target interactions, reductions in off-target transcripts are generally less than observed for intended targets. Thus, their presence will not automatically result in a phenotype, even in cases where the off-target transcripts are relevant to the biological question under investigation. To examine the influence of off-target effects on screen results specifically, we evaluated the two siRNAs for each of the top five gene targets responsible for a decrease in cell viability as individuals (Figure 4c). For 4 of the 5 targets, both siRNAs yielded at least a modest decrease in cell viability, suggesting that the phenotype is target dependent. For RRM2, only one siRNA appeared active, leaving open the possibility that activity resulted from off-target effects. However, the inactive RRM2 siRNA was less capable of reducing RRM2 mRNA levels (Supplementary Table 4). Evaluation of additional siRNAs against all targets, especially RRM2, would be necessary to confirm target-specific effects with greater confidence. It is important to note that false positives arising from off-target effects

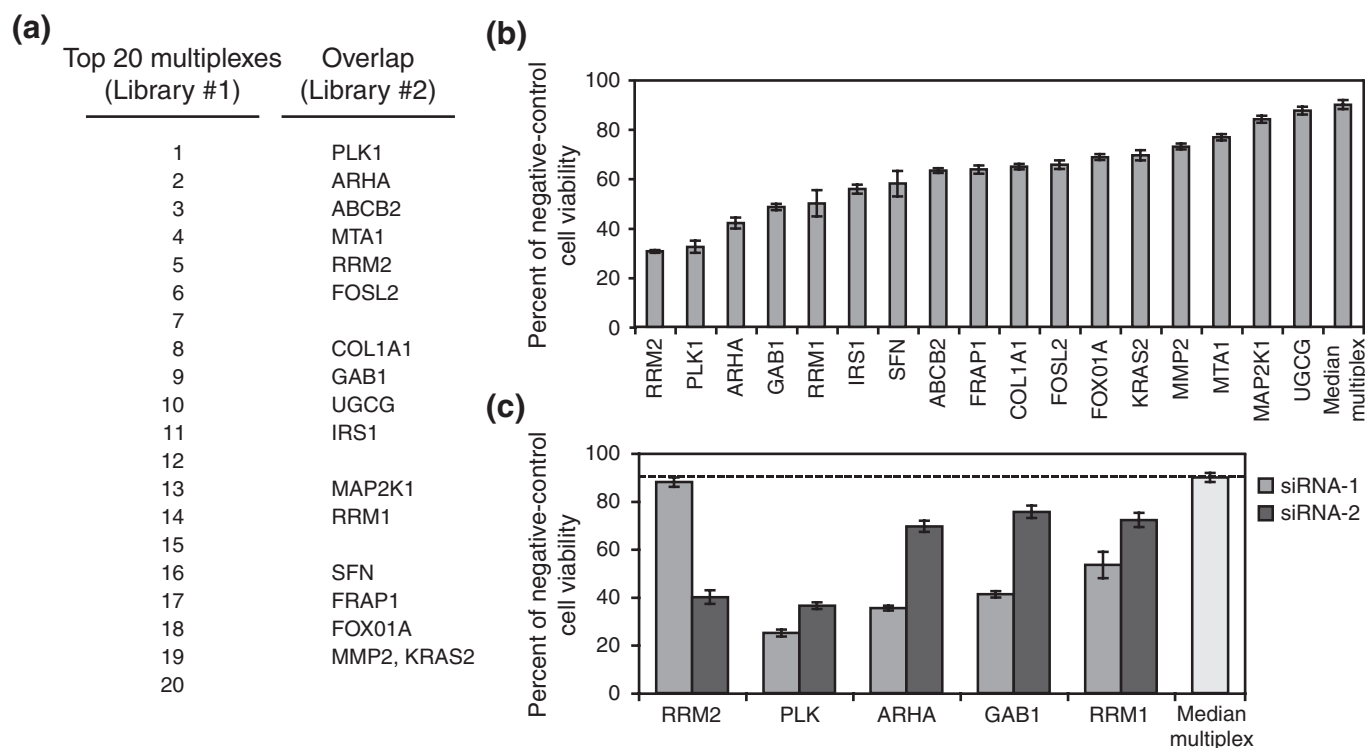


Figure 4. A dual library screening strategy for the identification of targets responsible for observed phenotypes. **(a)** The identified members of active siRNA multiplexes affecting cell viability. These target genes were identified by cross-referencing the components of the top 20 down-regulating multiplexes within library #1 with common components found in the top 20 down-regulators within library #2. **(b)** Validation of the identified targets as down-regulators of cell viability in an independent assay. All values are reported as the percent viability of cells transfected with a negative-control siRNA. As an additional reference, a multiplex found to approximate the median value in all three screens of library #1 was also used. **(c)** Evaluation of both siRNAs against the top five gene targets individually. The experiment was performed as described in **(b)**. The activity of *RRM2* siRNA-1 did not differ from that of the median multiplex. For each target, the activity of siRNA pairs was approximately equal to that of the more active siRNA (compare **(b)** with **(c)**).

would occur regardless of whether a multiplexed or arrayed format were used. Multiplexes would exhibit a higher rate of false positives only in cases where a sum effect between multiplex members results in a measurable phenotype. A dual library strategy would help to identify these situations. For example, the multiplexes ranked 12 and 15 in Figure 4a are the same as the deconvoluted multiplexes E and F in Figure 3c. Notably, the advent of siRNA chemical modifications that reduce off-target interactions (32) and the development of new algorithms that include a more current understanding of off-target interactions (e.g. avoiding microRNA-type interactions between siRNAs and untargeted 3' UTRs (33)) may make the use of a multiplexed strategy even more appealing.

Through the use of a library comprising randomly generated multiplexes of siRNAs that target multiple genes, we were able to streamline a siRNA-based screen substantially. Both the identification of established anti-cancer therapeutic targets and the identification of siRNAs ranking highest in an analogous arrayed screen help to validate this type of approach. Furthermore, we found that the number of downstream experiments required for validation could be minimized through the use of a carefully constructed second library that facilitated identification of key targets with only limited deconvolution. Although we conducted a screen using

multiplexes of siRNAs directed against three gene targets, presumably screens could also be conducted with siRNAs multiplexed in larger numbers, as we have demonstrated that at least six targets can be co-silenced. To explore this further, we conducted a screen comprising six gene multiplexes in MDA-MB-231 cells (data not shown). We found that 6 of the 8 multiplexes that ranked highest in terms of a decrease in cell viability contained gene targets ranked among the top 10 of the arrayed screen, suggesting that it is plausible to screen larger multiplexes. However, analogous to similar strategies using compound libraries, multiplex size needs to be balanced with the number of expected hits in order to limit the number of downstream experiments necessary for deconvolution. For example, an optimal mixture size of 15–20 compounds is predicted for random chemical libraries exhibiting hit rates of 0.2–0.35%, whereas biased compound libraries, with hit rates greater than 5%, have optimal pools sizes of less than five compounds (34). Consequently, screens exhibiting high hit rates may not be amenable to a random multiplexed strategy. For example, screens biased towards using siRNAs against related targets, such as the human kinome, can have very high hit rates (8,13). However, in other contexts, screens conducted with large unbiased siRNA libraries would be expected to exhibit low hit rates. For example, an shRNA library screen

corresponding to ~8000 genes identified only six significant modulators of p53-dependent cell proliferation arrest (4). Thus, a random multiplexed strategy may be applicable to a wide variety of studies with optimal multiplex size ultimately dependent upon expected hit rates as well as other aspects of the assay and its aims. Altogether, this study suggests that the screening of randomly multiplexed siRNAs may provide an important avenue towards the rapid identification of candidate gene targets for downstream functional analyses and may also be useful for the rapid identification of positive controls for use in novel assay systems. A multiplex-based strategy may be especially applicable in cases where assay costs, platform limitations and other practical issues are prohibitive.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR online.

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Conflict of interest statement. Dr. E. Lader is an employee of Qiagen and is currently conducting research sponsored by this company.

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