Reverse Transfection Format
siRNA libraries enable rapid and economical RNAi screening

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Introduction
High-throughput RNA interference (RNAi) screening is an effective strategy for studying gene function and characterizing genes within biological pathways of interest. However, large-scale arrayed screens are time consuming and require expensive instrumentation for automation and liquid handling. Conventional transfection, or forward transfection, is a common technique for delivering siRNA into cells for gene silencing. In this standard approach, cells are plated one day prior to treatment, at which time siRNA is complexed with a transfection reagent and then added to the pre-plated cells. In high throughput assays, the multiple liquid handling steps of the forward transfection workflow require automation for uniformity, which can be cost prohibitive. Many screeners utilize reverse transfection, where cells are added to arrayed siRNA-transfection reagent complexes. This method shortens the experiment by one day by circumventing the need to pre-plate cells and eliminates the requirement for medium replacement with transfection medium. However it still requires high-throughput liquid handling to array the siRNAs.

Dharmacon Reverse Transfection Format (RTF) siRNA libraries are SMARTpool™ siRNA reagents pre-arrayed in tissue culture-ready 96-well plates, which eliminate the need for any liquid handling of siRNA and pre-plating cells (Figure 1). This streamlines the transfection workflow, permitting rapid and economical screening without the requirement of extensive high-throughput automation. The libraries are available as pre-defined gene sets for human and mouse, targeting genes involved in phylogenetically related pathways or gene families. These focused libraries further simplify the scope and scale of the screen to higher-probability hits. In addition to pre-defined libraries, custom libraries are also available.

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Figure 1. Reverse transfection with RTF Libraries. Transfection rehydration solution is prepared (Step 1) and used to rehydrate and complex the pre-arrayed siRNAs with transfection reagent in multi-well tissue culture plates (Step 2). After a short incubation, cells are directly added to the wells (Step 3), and 24 to 96 hours post-transfection analyzed in a phenotypic assay of interest (Step 4). Libraries are available for human or mouse as ON-TARGETplus™ or siGENOME™ siRNA reagents and are supplied in six replicates ready for a 50 µM transfection of siRNA. Additional plate replicates are available. Each plate also contains negative and positive control siRNA reagents, as well as empty wells reserved for reagents of choice and untransfected controls.
This application note demonstrates a workflow for successful identification of target genes involved in a pathway of interest. We used the human Dharmacon™ ON-TARGETplus™ RTF SMARTpool siRNA Library targeting 240 genes involved in the DNA damage response network to perform a high-content analysis screen in human A549 cells. Upon silencing of DNA damage response genes, cells were analyzed for phosphorylation of histone H2AX, an early marker of DNA damage (Figure 2).

Materials and methods

Reverse transfection
DharmaFECT™ 1 transfection reagent (Cat #T-2001) diluted in DharmaFECT Cell Culture Reagent (Cat #B-004500-100) was added to ON-TARGETplus RTF plates in a total volume of 25 μL per well to rehydrate and complex siRNA for 30 minutes. For transfection optimization, DharmaFECT 1 was added to DharmaFECT Cell Culture Reagent at a range of concentrations and 25 μL added to each well of Human ON-TARGETplus RTF Optimization Plates (Cat #H-102200) for rehydration and complexing. For the screen and hit follow-up transfections, 0.15 μL DharmaFECT 1 and 24.8 μL DharmaFECT Cell Culture Reagent were added to each well of Human ON-TARGETplus RTF DNA Damage Response Library plates (Cat #H-106005) or custom-ordered ON-TARGETplus RTF plates, respectively, for rehydration and complexing. RPA2 positive control siRNA reagent (6.25 pmol) was manually spotted into vacant wells of RTF plates and desiccated before rehydration. Immediately following rehydration and complexing in each experiment, 3000 A549 cells (ATCC Cat #CCL-185) suspended in 100 μL full-serum medium were added to each well. Final concentration of siRNA in each well was 50 nM. Cells were incubated for 72 hours at which time the viability assay was performed and RNA harvested for RT-qPCR, or cells were fixed and stained for high-content analysis. Untreated cells were not exposed to siRNA or transfection reagent. Mock samples were not exposed to siRNA but were treated with transfection reagent. ON-TARGETplus Non-targeting Control Pool (Cat # D-001810-10) was used as a negative control for all transfections. All conditions were performed in biological triplicate.

Cell viability assay
Resazurin (Acros Organics Cat #189900250) was added to cells 72 hours post-transfection in culture medium to final concentration of 1 ng/mL and incubated under cell culture conditions until color shifted from deep blue to purple (~2 hour). Colorimetric data were collected using a Wallac 1420 VICTOR2™ (Perkin Elmer Cat #1420-832) and the reported value was normalized to untreated cells.

RT-qPCR
RNA was isolated 72 hours post-transfection using SV 96 Total RNA Isolation System (Promega Cat #Z3505), reverse transcription was performed using Thermo Scientific™ Maxima™ First Strand cDNA Synthesis Kit (Cat #K1642) and gene expression analysis was performed using Thermo Scientific™ Solaris™ qPCR Gene Expression Assays (see Table 1 for Cat #) and Master Mix (Cat #AB-4350), according to manufacturer’s protocols. qPCR data was collected using a Roche LightCycler® 480 and 384-well white plates (Roche Cat #04729749001). Relative expression was calculated using a ΔΔCq method (2) in which target expression is normalized to reference gene expression and then to expression in non-targeting control.

Table 1. Solaris qPCR Gene Expression Assays.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene ID</th>
<th>Catalog Number</th>
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<tbody>
<tr>
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<td>2597</td>
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<td>RRM2</td>
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<td>AX-010379-00</td>
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</table>

Cell staining and high-content analysis
All steps were performed at room temperature. Washing was performed using a multi-well plate washer. Wash Buffer, Wash Buffer II, Permeabilization Buffer and Blocking Buffer reagents from Thermo Scientific Buffer Kit

Figure 2. DNA damage response pathway. Double strand breaks arise as a result of DNA damaging agents or spontaneously as a result of a variety of cellular processes including metabolism, recombination and transposition. Double strand breaks are also created when DNA replication forks stall or approach a nick in template DNA. H2AX (a constituent of core histone complexes) surrounding double strand breaks are rapidly phosphorylated, inducing a set of responses through the DNA damage signaling network, including DNA repair, cell cycle arrest and apoptosis. RPA2, a subunit of replication protein A, was used as a positive control in the screen. The RPA complex binds single stranded DNA in both DNA replication and homologous recombination repair structures. RPA knockdown destabilizes replication forks and inhibits homologous recombination repair, resulting in an accumulation of double strand breaks and hence phosphorylated H2AX (1).
2 (Cat #8422000) were used. Cells were fixed with 100 μL per well 16% paraformaldehyde (Electron Microscopy Sciences Cat #15710) diluted four-fold in Wash Buffer for 15 minutes in a fume hood, and then washed twice with 100 μL Wash Buffer per well. Cells were then permeabilized for 15 minutes with 100 μL Permeabilization Buffer, washed twice with 100 μL Wash Buffer, and then blocked with 100 μL Blocking Buffer for 15 minutes. Samples were then incubated with Thermo Scientific pH2AX Primary Antibody (Cat #1860938) diluted in Blocking Buffer 1:1000 (50 μL per well) for one hour and then washed twice with 100 μL Wash Buffer II and twice more with 100 μL Wash Buffer. Thermo Scientific DAPI Nuclear Counterstain (Cat #62248) and DyLight550-conjugated secondary antibody (Cat #84540) were diluted 1:2000 and 1:500, respectively, in Blocking Buffer and 50 μL per well of this solution was incubated with the samples for 45 minutes. Samples were washed twice with 100 μL Wash Buffer II and twice more with 100 μL Wash Buffer. Fresh Wash Buffer was replaced and plates were scanned with Thermo Scientific™ ArrayScan™ VTI (Cat #NO1-0002). The CellCycle. V4 BioApplication was used with fixed exposure times and background correction with a minimum of 500 cells analyzed per well. Total intensity data was collected for both DAPI and phospho-H2AX channels. To calculate percent positive phospho-H2AX cells, the mean and standard deviation of the total intensity was calculated for more than 1000 NTC-treated cells. The average plus two standard deviations was set as the threshold for a positive cell. The percent positive cells per well was calculated for the NTC wells and for all other wells. If the average of the percent positive cells for the replicates for a particular target exceeded two-fold percent positive cells compared to NTC, this target was deemed a primary hit.

Results and Discussion:
Optimizing key steps in the reverse transfection workflow is necessary for reproducible identification of primary hits. An outline of the reverse transfection screen workflow and the time required to perform each step is presented in Figure 3. Optimizing the transfection conditions is necessary because cell viability and efficiency of target silencing are cell-type dependent and greatly affected by cell density and transfection reagent concentration. After finding an appropriate cell plating density for the high-content assay (60–80% confluency at the time of data collection), the transfection was further optimized for appropriate concentration of DharmaFECT transfection reagent using RTF Optimization Plates, which are provided as a set of three plates that contain pre-dispensed positive and negative control siRNAs (Figure 4A). RTF Optimization Plates facilitate negative control selection, as they contain individual and pooled non-targeting control siRNAs. Multiple DharmaFECT formulations can also be tested using these plates, allowing selection of the most efficacious and least toxic transfection reagent formulation for a given cell type. Transfection optimization for A549 cells identified that 0.15 μL transfection reagent per well caused efficient knockdown of PPIB positive control at the desired cell density with no adverse affect on cell viability (Figure 4B).

For optimizing the high-content assay, RPA2 was used as a positive control. RPA2 silencing has been shown to induce DNA damage response (3). ON-TARGETplus reagents silenced RPA2 mRNA by 96% (Figure 5A), which resulted in an increase of cells stained with phospho-H2AX antibody in the high-content assay (Figure 5B and C). The Z’ factor of 0.79 indicates that the phenotypic assay is robust (low variability and high dynamic range) and suitable for siRNA screening.

The DNA Damage Response RTF collection was transplanted in biological triplicate on the same day by one researcher without the use of high-throughput liquid handling instrumentation or robotics. Only a hand-held Thermo Scientific™ Matrix™ 12-Channel Electronic Pipette (Cat #2014) was used to aid in throughput. Seventy-two hours post-transfection, cells were fixed and stained for high-content analysis. Thirty-five primary hits, which exhibited a reference gene. Cells were exposed to transfection reagent only to assess its effect alone (MOCK).

**Figure 3. DNA damage response RTF screen workflow.** Transfection optimization is necessary to find the best conditions (transfection reagent concentration and cell density) for efficient gene silencing in the cell line of choice (Step 1). Cell plating density is determined such that confluency is 60–80% at the time of high-content analysis. Lipid concentration is optimized based on this pre-determined plating density. Assay optimization can be facilitated by selecting and testing positive controls (Step 2). The RTF siRNA library is then transfected, data collected and primary hits are identified (Step 3). Subsequently, hit confirmation and follow-up are conducted to validate screen results and further investigate the targets’ role in the context of the biological system (Step 4). The times required to perform these steps for the DNA damage response screen are indicated.

**Figure 4. Transfection optimization with RTF Optimization Plates.** A. RTF Optimization Plates are configured to support determination of optimal RTF transfection conditions. The dose curve carried out with the selected DharmaFECT transfection reagent formulation is indicated as μL/well. B. For A549 cells at 3000 cells per well, 0.15 μL DharmaFECT 1 per well yielded 79% knockdown with no adverse affect on cell viability. Cell viability was determined using a resazurin assay (data normalized to untreated cells (UT)) and relative gene silencing was assessed by RT-qPCR (data normalized to both non-targeting control siRNA (NTC) and GAPDH as a reference gene). Cells were exposed to transfection reagent only to assess its effect alone (MOCK).
Figure 5. RPA2 as a positive control for assay optimization.

A. Reverse transfection of siRNA targeting RPA2 yields efficient gene silencing. Cell viability was determined using a resazurin assay (data normalized to untreated cells) and relative gene silencing was assessed by RT-qPCR (data normalized to both non-targeting control siRNA and GAPDH as a reference gene). B. Immunofluorescent images show an increase in the number of cells stained with anti-phospho-H2AX antibody in the sample transfected with RPA2 siRNA compared to non-targeting control siRNA. C. High-content analysis indicates an increase of the percent of phospho-H2AX positive cells to 37% compared to less than 1.4% in the control cell populations (UT: Untreated, MOCK: lipid only, NTC: Non-targeting control pool and PPIB). The Z’ factor of the positive control is indicated on the graph.

Figure 6. RTF screen primary hit identification. The DNA Damage Response RTF library, comprised of siRNAs targeting 240 DNA damage response genes, was transfected into A549 cells in biological triplicate. The library includes individual and pooled non-targeting siRNA negative controls and a pooled positive control targeting PPIB as a transfection control. Additionally, RPA2 siRNA was transfected as an assay positive control. The 35 primary hits, which exhibited percent phospho-H2AX positive cells two-fold or greater over non-targeting control, are shown separately below the entire data set.

Figure 7. Hit follow-up analysis: A. Efficient gene silencing was observed by RT-qPCR (normalized to both GAPDH reference gene and non-targeting control) for all four targets tested. B. Cell cycle profiles of A549 cells transduced with non-targeting siRNA control pool and siRNA reagents targeting POLA1 or RRM2 are displayed as histograms of number of cells as a function of DNA content. Cells transfected with siRNA targeting POLA1 display an increase of cells' DNA content characteristic of G2/M phase block, while cells transfected with siRNA targeting RRM2 contain a high fraction of sub-G1 apoptotic cells.
percent positive cells two-fold or greater over non-targeting control, were identified (Figure 6). These hits were identified without an external DNA damaging agent in the screen and hence are targets involved in response to intrinsic, routine genomic instability and cellular stress. Additional targets may be revealed from a screen performed in the presence of a DNA damaging agent.

Follow-up was performed on the RPA2 positive control plus three targets that met the criteria for a hit: CHEK1, RRM2 and RPA1. Efficient gene silencing was observed for all four targets (Figure 7A). It is recommended to further confirm the phenotype with additional siRNA reagents (a different Dharmacon™ SMARTpool™ or multiple individual siRNAs). Additionally, siRNA specificity can be ultimately validated with a phenotype rescue experiment (4).

In addition to measuring the level of phosphorylation of histone H2AX as a primary assay, cell cycle and cell number analyses were performed on the ArrayScan VTI using DAPI dsDNA stain. To show the distribution of DNA content within cell populations, data were plotted as a histogram of number of cells as a function of nuclear stain intensity (Figure 7B). Knockdown of POLA1, the catalytic subunit of DNA polymerase A, impeded transition to G2 and resulted in an accumulation of cells in the S phase and a decrease in cell count by 65%. Knockdown of RRM2 resulted in a substantial increase of sub-G1 cells compared to control, indicative of cells undergoing apoptosis. RRM2 catalyzes the conversion of ribonucleosides to deoxyribonucleosides, which are necessary for DNA synthesis and repair (5). RRM2 is only expressed in the late growth phase and early synthesis phase, and is degraded in late synthesis phase (6). Over-expression of RRM2 leads to tumorigenesis (7), indicating a lack of cell cycle control, while siRNA-mediated gene silencing of RRM2 is reported to reduce cell proliferation (8). These examples demonstrate the utility of multi-parametric data acquisition possible with ArrayScan VTI for assessing multiple phenotypes within the same experiment.

Conclusions
RTF siRNA Libraries utilize potent SMARTpool siRNAs in a convenient, single-use 96-well format that streamlines siRNA screening. From optimization to primary hit identification, the screen of 240 genes was completed in three weeks without costly robotics. The results of the DNA damage response screen demonstrate the power of using high-content screening to assess multiple phenotypic changes due to specific RNAi-mediated gene silencing.

References