A 1,536-Well Ultra-High-Throughput siRNA Screen to Identify Regulators of the Wnt/β-Catenin Pathway

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ABSTRACT
High-throughput siRNA screens are now widely used for identifying novel drug targets and mapping disease pathways. Despite their popularity, there remain challenges related to data variability, primarily due to measurement errors, biological variance, uneven transfection efficiency, the efficacy of siRNA sequences, or off-target effects, and consequent high false discovery rates. Data variability can be reduced if siRNA screens are performed in replicate. Running a large-scale siRNA screen in replicate is difficult, however, because of the technical challenges related to automating complicated steps of siRNA transfection, often with multiplexed assay readouts, and controlling environmental humidity during long incubation periods. Small-molecule screens have greatly benefited in the past decade from assay miniaturization to high-density plates such that 1,536-well nanoplate screenings are now a routine process, allowing fast, efficient, and affordable operations without compromising underlying biology or important assay characteristics. Here, we describe the development of a 1,536-well nanoplate siRNA transfection protocol that utilizes the instruments commonly found in small-molecule high throughput screening laboratories. This protocol was then successfully demonstrated in a triplicate large-scale siRNA screen for the identification of regulators of the Wnt/β-catenin pathway.

INTRODUCTION
As technologies in the field of RNAi high-throughput screening (HTS) mature, the focus is now shifting to designing and implementing screening strategies that provide high-quality data at the primary screening level. It is hoped that these new efforts reduce false discoveries stemming from technical and biological variances and from off-target effects. High content assays that generate phenotypic profiles instead of single measurements have been used to help identify hits that are weak in potency but biologically relevant. The development of miniaturized screening platforms with higher throughput could further improve the selection of relevant hits from siRNA by allowing fast and affordable production of replicate data in the primary screen.

High-throughput genomic screening using spotted glass microarray technologies provides substantial throughput increase and has already proven successful. However, the types of assays that can be used in microarray format are mostly limited to intracellular fluorescent readouts. Alternatively, microplate-based methods are not hindered by readout limitations and are compatible with many liquid-handling instruments typically available in most HTS

ABBREVIATIONS: BAR, β-catenin activity reporter; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; GPCR, G-protein coupled receptor; HTS, high-throughput screening; MAD, median absolute deviation; RT, room temperature.
laboratories. The throughput of microplate-based screening depends on plate well density and available HTS infrastructure. Previously, we developed a streamlined siRNA transfection protocol in a 384-well plate format and demonstrated a significant increase in transfection throughput in a genome-scale siRNA screen. However, assay miniaturization technologies for small-molecule HTS has advanced well beyond 96- and 384-well microplate formats, and many liquid-handling instruments currently used for HTS are now capable of routine handling of 1,536-well nanoplates. Here we demonstrate the feasibility of RNAi screens in 1,536-well nanoplate format using common liquid-handling instruments developed for small-molecule ultra-HTS (uHTS). Specific technical issues related to siRNA screening, including lipid-based transfection and long duration of assays, can be overcome with assay development and the use of appropriate automation equipment.

To demonstrate the practicality of the newly developed siRNA transfection protocol for 1,536-well nanoplate uHTS, an siRNA screen of the human druggable genome (~6,700 genes) was run in triplicate to identify novel regulators of the Wnt/β-catenin pathway. This pathway represents a GPCR-mediated cell signaling pathway involved in development, cancer, tissue remodeling, bone biology, and stem cell renewal. Eighty percent of human colorectal cancers, the second leading cause of cancer death in the United States, are associated with various mutations in the APC gene, a negative regulator of β-catenin. A β-catenin activity reporter (BAR) plasmid was engineered into the DLD1 colorectal tumor cell line with a loss-of-function mutation in the APC gene that results in constitutively active β-catenin. The luciferase reporter assay was adopted to develop an automated screening protocol for 1,536-well nanoplate siRNA uHTS, taking advantage of a large assay window and the ease of miniaturization because of a relatively small number of steps in the assay protocol. The goal was to identify the genes that regulate Wnt/β-catenin pathway as potential new targets for cancer therapy.

MATERIALS AND METHODS

Cell Line and Culture

DLD1 cells were stably transduced by lentiviral infections with a BAR that drives firefly luciferase expression from a promoter containing the LEF/TCF binding sites and a Renilla luciferase for the purpose of normalization to cell number. Cells were cultured in Dulbecco’s modified Eagle's medium (DMEM; catalog no. 11965; Gibco) with 10% fetal bovine serum (FBS; catalog no. 10082-147; Gibco).

Control siRNAs

Each screening plate contained 2 positive control siRNA pools, including the following β-catenin (pool of 3 siRNAs): 5′-CUAUCCUGUCGUCCUCUAGUA-3′, 5′-CUGUUGGAAGUGAAGCUAGAA-3′, and 5′-GAGGACCUCUAUCUACGAA-3′; APC: 5′-GGGUUCUAUCAUGGAAUU-3′, 5′-GAUGGAAUGUGCUUUAGAA-3′, and 5′-CAGAGAAGUA CUGGUAUAU-3′. As a negative control, a luciferase siRNA (5′-CGUA CGCGGAUACUUCGA-3′) was used.

siRNA Library and Screening

A total of 6,729 siRNA pools (an equimolar mix of 3 siRNA duplexes per pool) representing the same number of genes were tested. This siRNA library was designed by Rosetta Inpharmatics and synthesized by Proligo. The siRNA transfection is performed as follows: 0.5 μL of 0.5 μM sample and control siRNA were plated by BioMek FX (Beckman Coulter) into 1,536-well polystyrene, white, sterile, TC-treated assay plate (catalog no. K1,536SWST; Kalypsys). About 1.5 μL of Lipofectamine RNAiMax (catalog no. 13778-150; Invitrogen), pre-diluted 1:100 in OptiMEM (catalog no. 31985-070; Invitrogen), was dispensed into the assay plate by BioRapTR (Beckman Coulter). Cells were dissociated with TrypLE Express Stable Trypsin–Like Enzyme (catalog no. 12605028; Invitrogen) and re-suspended at a density of 1.43 × 10^5 cells/mL in the plating medium (DMEM, 10% FBS; see above for details). After 30 min of incubation of the siRNA/RNAiMax mixture at room temperature (RT), 7 μL of assay media or DLD1 BAR cell suspension (1,000 cells per well) was dispensed into the assay plate using a Multidrop Combi (Thermo Fisher Scientific). The final siRNA concentration was ~28 nM. Cells were incubated for 72 h at 37°C and 5% CO2 in an incubator with >95% relative humidity, covering the plates with a plastic lid, and minimizing opening of the incubator door to maintain high humidity and prevent evaporation. After the 72 h of incubation, 7 μL of the medium was aspirated off from the wells using a Kalypsys 1,536-Well Washer/Dispenser. Three microliters of Luciferase substrate (catalog no. PRE2940; Promega Dual-Glo) was added to the assay plate by BioRapTR and incubated for 15 min at RT, and firefly luciferase luminescence signal was measured by ViewLux plate reader (60 s exposure; Perkin Elmer). Three microliters of Stop&Glo substrate (catalog no. PRE2940; Promega Dual-Glo) was next added to the assay plate (BioRapTR), followed by a 15 min of incubation at RT and signal from Renilla luciferase luminescence measured using the ViewLux (120 s exposure).

Data Processing

A ratio of firefly luminescence (β-catenin activity) to Renilla luminescence (cell viability) raw luminescence counts was calculated for each well and log 2–transformed. Further calculations were done at plate-wise level. The median of log 2–transformed ratio for the sample field region was calculated and subtracted from the individual well value. In turn, this difference in log 2–normalized values was divided by the median absolute deviation (MAD) of the log-normalized firefly luminescence values of the sample field to generate a plate-wise MAD-based Z*-score value, as shown below:

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RESULTS

The 1,536-Well Nanoplate Miniaturization of an siRNA Assay

An engineered cell line that harbors a BAR plasmid was used to identify siRNAs that inhibit or activate BAR activity in the DLD1 colorectal tumor cell line with a loss-of-function mutation in the APC gene, leading to a constitutively active β-catenin.16,17 This BAR-Luc DLD1 assay was robust in a 384-well format, and cells were efficiently transfected by lipid-based methods, as measured by >95% decrease in BAR activity by 2 different sequences of siRNA to the β-catenin gene (Fig. 1). Extending from the successful validation in the 384-well microplate format, we explored the feasibility of screening this assay in a 1,536-well nanoplate format to implement a large-scale siRNA screen in triplicate. An automation protocol for siRNA transfection in 1,536-well nanoplate format was developed in which siRNA solutions were transferred from 384-well siRNA source plates into 1,536-well assay plates, followed by the addition of prediluted Lipofectamine RNAi Max transfection reagent (Fig. 2A). A key challenge was to reliably transfer siRNA in a very low volume (0.5 or 1 μL) and effectively mix it with transfection reagent to yield high transfection efficiency. The siRNA transfer in this protocol represents dry transfer since aqueous siRNA solution is transferred to dry, empty wells of 1,536-well nanoplate. Accuracy and consistency in such dry transfer of a very small volume are difficult to achieve and require fine-tunings of the robotic pipetting process. Briefly, tips were lowered down to lightly touch the bottom of the well, 0.5 or 1 μL of liquid dispensed, followed by an air blowout of 2 μL, and tip exiting from the well. After 20 min of incubation of the siRNA/transfection mixture, a cell suspension (1,000 cells/well) was added directly into the siRNA/transfection reagent mixture (reverse transfection). Both 1.0 and 0.5 μL transfer of siRNA solution (total volume of siRNA/lipid transfection mixture was 2 μL) resulted in high and consistent transfection efficiency, as judged by more than 95% decrease in the BAR activity with β-catenin siRNA (tested siRNA concentrations: 7–56 nM; Fig. 2C). To keep the volume of the medium in the 1,536-well nanoplate to a minimum and prevent potential overflow and cross contamination, we decided to use 0.5 μL siRNA transfer in subsequent experiments. The comparison of transfection conditions for 384- and 1,536-well plates is summarized in Table 1. Other features of the protocol included the elimination of the steps mixing siRNA/transfection reagents and centrifugation after each liquid transfer, as data indicated that they did not affect the assay outcome (not shown). This streamlined the overall protocol and helped further increase transfection throughput. We also tested various storage conditions for the siRNA preplated in 1,536-well nanoplates. We compared those plates that were kept at ambient temperature and humidity for 1 h, 4 h, or kept overnight in a plastic bag in the 4°C refrigerator, with or without plate sealing. All tested storage conditions showed similar levels of siRNA transfection efficiency (data not shown).

In the final protocol, a Beckman Biomek liquid transfer instrument was used to transfer 0.5 μL of sample or control siRNA solution from four 384-well plates to one 1,536-well nanoplate (4:1 compression; Fig. 2B). The 1,536-well nanoplates preplated with siRNA solutions

\[ Z'^{-}\text{score}(\text{nLogFF}) = \frac{(\text{Log Normalized Firefly LUM [All wells]}) - \text{Med}[\text{Log Normalized Firefly LUM [Sample]}]}{\text{Mad}[\text{Log Normalized Firefly LUM [Sample]}]} \]
were spun by brief centrifugation, and Lipofectamine RNAi MAX (prediluted 1:100 in OptiMEM) was added to form siRNA/transfection reagent complex for 20 min at RT. Cells (columns 3–48) or the medium (columns 1–2) were then dispensed with a BioRapTR bulk dispenser (Beckman Coulter). The final siRNA screening concentration was 28 nM. Seventy-two hours posttransfection, reporter assays were performed using the Promega DualGlo kit as described in the Materials and Methods section. Data were acquired with a ViewLux CCD-based plate imager (exposure time: firefly = 60 s; Renilla = 120 s). This protocol produced good transfection efficiencies as well as good assay quality data, without systematic edge effects due to evaporation (Fig. 3).

The 1,536-Well Nanoplate siRNA uHTS

A druggable genome siRNA collection (~6,700 genes targeted by pools of 3 siRNA duplexes per gene) was used to test the performance of the 1,536-well nanoplate assay protocol. On a Thermo CRS robotic platform equipped with the instruments described above, the DLD1-BAR cells were transfected in triplicate from six 1,536-well siRNA source plates that were 4-to-1 precompressed from twenty-four 384-well plates using a Beckman FX 384-well pipettor. Each 1,536-well assay nanoplate was processed every 2 min and each replicate every hour with a fresh batch of DLD1-BAR cells. The druggable genome siRNA screen ran successfully in an efficient manner that allowed each step in the protocol to run at a precise timing and eliminated idling time for the robotic arm. The new 1,536-well siRNA transfection protocol helped achieve data quality that was high and consistent across triplicate runs (Fig. 4A). Transfection efficiency and Z’-factors were also consistently high from plate to plate throughout the screen (average 92% and 0.68, respectively; Fig. 4B, C).

Correlation analysis showed that the screen results were highly reproducible between the combinations of paired replicates (Fig. 5). Correlation coefficients (R²) were >0.77 when sample siRNAs were compared, and >0.90 when both sample and control siRNAs were compared. The higher R² for the latter is due to greater data dynamic

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**Fig. 2.** (A) Diagram for 1,536-well siRNA transfection protocol. About 0.5 μL of siRNAs solution was dispensed from four 384-well microplates into a single 1,536-well assay nanoplate using a Biomek. The 1,536-well assay nanoplates are then centrifuged to collect liquid to the well bottoms and used immediately or kept at 4°C for later use. About 1.5 μL of diluted Lipofectamine RNAi MAX transfection reagent (1:100 in OptiMEM media) is dispensed into the assay plate using BioRapTR (Beckman Coulter). Thirty minutes after the formation of siRNA:RNAi MAX at ambient temperature, transfection reagent (1:100 in OptiMEM media) is dispensed into the assay plate using BioRapTR (Beckman Coulter). About 0.5 μL of diluted Lipofectamine RNAi MAX transfection reagent (1:100 in OptiMEM media) is dispensed into the assay plate using BioRapTR (Beckman Coulter). The final siRNA concentration was 28 nM. Seventy-two hours after transfection, dual luciferase assays (firefly luciferase for signal and Renilla luciferase for normalization) were performed according to manufacturer’s protocol (Promega), two hours after transfection, dual luciferase assays (firefly luciferase for signal and Renilla luciferase for normalization) were performed according to manufacturer’s protocol (Promega), followed by data acquisition with ViewLux CCD-based plate imager (exposure time: firefly = 60 s; Renilla = 120 s). (B) siRNA preplating from 384-well microplates into 1,536-well nanoplates. About 0.5 μL of siRNAs was transferred from four 384-well microplates into a single 1,536-well nanoplate using a dry transfer technique. Percent coefficient of variation (CV) for 0.5 μL transfer was <5%. (C) siRNA preplating and the quality of transfection. About 0.5 μL of 0.5 μM Luc siRNA (a non-targeting variant as negative control), β-catenin siRNA (as either a pool of 3 individual siRNAs or 1 of the 3 singles), and water (mock transfection) were transferred from 384-well microplate into 1,536-well nanoplate, and the rest of assay was performed as described above. High transfection efficiencies were obtained consistently with all tested β-catenin siRNA concentrations (7–56 nM), as judged by >90% decrease in the BAR reporter activity. Results were derived from n = 48, and error bars indicate standard deviation. Same results were obtained dispensing 1 μL of siRNA solution. BAR, β-catenin activity reporter. Color images available online at www.liebertonline.com/adt.
ranges with control siRNAs. These numbers represent a significant improvement from previous siRNA screens done in 384-well microplates, where $R^2$ values of 0.5–0.6 were typical.\textsuperscript{10,11} This improved correlation is partly attributed to the much shorter duration of the screen, which helps maintain the high quality of the cells.

### Hit Selection

To select hits from the screen, we calculated the $Z^*$-scores as described in Materials and Methods.\textsuperscript{11} To better appreciate the power of replicate siRNA screen, 2 different approaches to hit selection were compared. In the first approach, we treated each replicate screen as if it was performed independently. Then, we selected hits if their $Z^*$-scores were /C0\textsubscript{3} or smaller. This exercise resulted in 30, 36, and 28 hits for replicates 1, 2, and 3, respectively (Fig. 6A). It was clear that approximately half the number of hits in 1 replicate were selected as hits in another replicate. This rather small degree of overlap between replicates was because, even though overall reproducibility was high between replicates (judging from high $R^2$ values), $Z^*$-score values for individual hits

### Table 1. Comparison of 384- and 1,536-Well Format Assay Protocols

<table>
<thead>
<tr>
<th>Plate Type</th>
<th>1,536</th>
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<tbody>
<tr>
<td>Cell number/well</td>
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<td>Transfection method</td>
<td>Reverse</td>
<td>Forward</td>
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<td>Regular plating, 1 µL wet transfer</td>
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<tr>
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<td>72 h</td>
</tr>
<tr>
<td>Transfection efficiency</td>
<td>&gt;90%</td>
<td>&gt;90%</td>
</tr>
</tbody>
</table>

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**Fig. 3.** Heat map view of the raw data from the Renilla (A), firefly (B), and normalized (C) luciferase activities from the CCD-based Viewlux images showed that there were no systematic edge effects. In a 1,536-well nanoplate, controls are located in columns 1–4 and 45–48: columns 1–2 are background signal with no cells; columns 3–4 are the mock control, cells treated with transfection reagent only; columns 45–46 contain several controls, including a nontargeting Luciferase siRNA used as a negative control, and siRNA to Axin and β-catenin, which are used as biological positive controls; and columns 47–48 contain nontreated cells. The 1,280 wells containing the siRNA test samples are located in columns 5–44. PLK1 indicates siRNAs to Polo-like kinase 1 imbedded in the sample field as additional controls for transfection efficiency as measured by reduced signal in the Renilla channel. In the images shown, darker color indicates higher luminescence signal. Color images available online at www.liebertonline.com/adt.
often crossed below or above the cut-off line ($Z^* = -3$) from 1 replicate to another. For example, USP46 siRNA had normalized $Z^*$-scores of $-2.86$, $-2.80$, and $-3.07$ from its 3 replicates, and therefore was declared a hit in 1 replicate but not in the other 2. On the other hand, NOX4 siRNA had $Z^*$-scores of $-2.36$, $-3.68$, and $-3.13$. Whether they represent real hits or not, this illustrates the high risk of selecting false-positives or -negatives with siRNA screens when they are performed in nonreplicate. In the second approach, hits were selected by more rigorous statistical measures (Fig. 6B). For each siRNA tested in triplicate, the median $Z^*$-score and $P$ value were calculated to draw a volcano plot. Twenty-seven siRNA hits were selected with a $Z^*$-score less than $-3$ (corresponding to approximately $<15.5\%$ activity) and $P < 0.05$. These hits overlapped with those hits selected in at least 2 replicates using the enumerative method (Fig. 6C). This does not suggest that 2 replicates are sufficient to identify all the statistically significant and strong hits because overlapping hits were different from 1 pair of replicates to another. Rather, it is more desirable to first determine the level of error rates (e.g., $P$ values of 0.05 or 0.01) and then select hits based on such a level of tolerance. Twenty-four genes with functional annotations were re-tested using additional siRNA pools of different siRNA sequences. Eight out of the 24 genes confirmed with 2 independent siRNA pools. Included in the 8 genes were $CTNNB1$ (β-catenin), which validates the hit selection approach, and other genes, which analysis for protein–protein interaction network by Ingenuity Pathway Analysis indicated belong to the HNF4α network ($PGM3$, $MKRN1$, and $GPR120$) and TGFβ1 network ($TK2$, $PPP1R16B$, $ITG11$).

**Fig. 4.** Screen results overview for triplicate druggable genome screen. (A) Scatter plot of log-transformed raw luminescence counts from ViewLux CCD plate imager for each well of each plate of a triplicate druggable genome sub-library screen. The plot shows remarkable absence of data fluctuations from plate to plate. Blue circles represent a Luc siRNA nontargeting negative control, and red circles correspond to the β-catenin siRNA, a positive control. (B) Plot of per plate $Z$-factor (screening window). $Z$-factors were calculated between wells transfected with Luc siRNA (negative control) and wells transfected with β-catenin siRNA, as background signal control. $Z$-factors of 0.6 or higher are considered to provide excellent screening window. (C) Plot of calculated average transfection efficiencies per plate. Transfection efficiencies were calculated using ($100 - \text{median RLU for β-catenin siRNA control wells)/medium RLU for Luc siRNA control wells.}$ It was consistently maintained at $>90\%$ throughout the plate runs. RLU, relative luminescence units. Color images available online at www.liebertonline.com/adt.
DISCUSSION AND CONCLUSIONS

siRNA HTS, in combination with other genomic and proteomic research tools, is fast becoming a powerful tool for mapping biological pathways and for identifying novel therapeutic targets and biomarkers. As more and more siRNA screening results are being published, it has become apparent that they are not straightforward for interpretation and validation, in part due to the scale and speed of data production, biological complexity, and the limitations of siRNA technology. Data noise and variability from these confounding factors usually result in high false discovery rates, and it is often the case that only a small number of strong hits are reproducibly confirmed in subsequent validation efforts. It is challenging to design and implement siRNA screens with low false discovery rates that enable the identifications of moderate but biologically relevant genes. Various strategies have evolved to address this issue, including carrying out primary screens in replicate, testing multiple individual siRNA duplexes for each target gene instead of pools of individual duplexes, performing multiplexed or high-content assays, and screening in multiple cellular backgrounds. Systematic implementation of these strategies is limited by the HTS infrastructure and screening throughput available in individual labs. By miniaturizing siRNA transfections and assays to 1,536-well nanoplate and utilizing existing HTS infrastructure, however, many of these strategies can be implemented in a timely and resource-efficient manner.

In this report, we have demonstrated an efficient uHTS siRNA transfection protocol in 1,536-well nanoplate format, using commercial lipid-based transfection reagents and common automation equipments. This new protocol was then successfully implemented in a rapid replicate screen of a druggable genome siRNA collection. Compared to 384-well microplate format, we have benefited significantly from a miniaturized assay protocol without compromising data quality. For example, siRNA transfection throughput has tremendously increased. In the past, using a 384-well plate protocol, we were able to transfected a genome-scale library of ~22,000 siRNA pools in 5–6 days over a period of 3 weeks in a nonreplicate screen. Using the current method, we estimate that genome-scale siRNA transfections in triplicate can be achieved in ~3 h. The use of expensive transfection reagents was dramatically reduced by 40-fold by miniaturization to 1,536-well plate, mostly by reducing dead volumes throughout the screen. Also, there was a significant reduction in the burden on cell culture operation, despite that the number of cells per well used in the 1,536-well plate was the same as in the 384-well plate. This lesser burden is largely because of the reduction in the usage of media, medium additives, and serum, as well as in the amount of wasted cells during screening process.

In addition to the obvious cost savings and enhanced productivity, an increased throughput helps produce higher quality data. For example, while it required us 3–4 batches of cells when screening a genome-scale library of ~22,000 siRNA pools in 384-well plates, with the 1,536-well nanoplate transfection protocol, such a scale of screening could be done with a single batch of cell suspensions, in triplicate, in a single day. A smaller number of batch runs for the entire screen in our case helped reduce data variation that are often associated with screening over several days. Also, because we completed the entire triplicate screen runs in a week, we were able to avoid a potential drift in cell line characteristics that can happen during an extended period of screening operations.

The ability to complete an RNAi screen at the high level of throughput that we have achieved without additional burden on the screening infrastructure could allow us to develop and implement various screening strategies that would have been challenging to practice in the past, yet are likely to identify better primary hits. In one strategy, by screening a genome-scale siRNA collection as single individual duplexes, instead of pools of duplexes, false-positive hits
due to off-target effects could be more readily detected and discarded.\textsuperscript{18} The elimination of off-target hits at the primary screen step would save from unnecessary validation experiments and help channel efforts toward true-positives regardless of their initial assay activity. Another strategy includes screening multiple cell lines to avoid idiosyncratic hits that are likely selected due to differences in cellular make-up related to, for instance, the integrity of genetic pathways and target mRNA expression levels. The success of these strategies applied to the primary screens that identified and validated novel modulators of the Wnt/\(\beta\)-catenin pathway has been reported elsewhere.\textsuperscript{16}

Despite the current challenges, an RNAi screen, especially when performed in replicate to reduce false discovery rates, can undoubtedly help us better understand the molecular mechanisms of human diseases and other physiological processes, and with continuous advances in screening technologies and strategies, it will remain a key tool in our technology portfolio for deciphering the functions of the human genome.

**AUTHOR DISCLOSURE STATEMENT**

No competing financial interests exist.

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