Automated Optimization of Cell Transfection

Abstract

Cell transfection is an essential technique for interrogating cellular pathways. Determining the optimal conditions for introducing nucleic acids into cells can be a time-consuming endeavor at the initiation of a cell biology experiment or screen. Here we demonstrate how the Biomek FX® Workstation can be used to identify the optimal conditions for siRNA transfection in a single experiment. Utilizing the enhanced multichannel selective tip pipetting, factorial combinations of transfection reagents and concentrations, cell number, and fluorescent oligonucleotide concentrations were plated in 384-well plates. This higher density plate format conserved reagents while also facilitating the replicate wells necessary to determine variability within a given condition. 24 hours after transfection, cells were stained with Draq7 and transfection efficiency and cell toxicity were measured on the SpectraMax i3 Multi-Mode Detection Platform with MiniMax 300 Imaging Cytometer. Automation enabled the miniaturization and execution of a complex optimization experiment, thereby reducing reagent costs and accelerating the timeline to meaningful transfection assays and screens.

Introduction

The introduction of exogenous nucleic acids into cells is an essential means of studying cell biology. Transient transfections can be used for screening the effects of overexpression (plasmid) or knockdown (siRNA) of genes as well as non-coding nucleic acids (miRNA mimics or inhibitors). By selecting for expression clones that have incorporated the construct of interest and a selection marker, stable lines can be generated for long-term studies or as reporter lines for screens. The challenge of transfection is to efficiently introduce the nucleic acid constructs into the cells while minimizing cell toxicity.

While alternatives such as electroporation can be used for transfecting challenging primary cells, chemical transfection agents are likely the most common method for transfecting established cell lines. These reagents typically package the nucleic acids in liposomes to allow them to pass through the cell membrane. These reagents can be developed for a given construct (i.e. plasmid vs. siRNA) and may be more or less effective for a specific cell type. However, identifying the most effective reagent for an untested cell line is only one aspect of transfection optimization. Transfection efficiency can vary dramatically based on the reagent concentration, the construct concentration, and the cell density. In addition, an excess of transfection materials can lead to cellular toxicity, so this condition must be monitored as well.

Here we describe an automated method that tests factorial combinations of differing amounts of various transfection reagents, oligonucleotides, and cells, to determine the optimal siRNA transfection conditions that maximize transfection efficiency while minimizing cell toxicity— all within a single experiment.

Materials and Methods

Cell Culture and Reagents: Colorectal carcinoma (HCT 116), pancreatic epithelioid carcinoma (PANC-1) and renal cell adenocarcinoma (ACHN) cells were cultured in McCoy’s 5A Modified Medium, Dulbecco’s Modified Eagle’s Medium, and Eagle’s Minimum Essential Medium respectively, each supplemented with 10% fetal bovine serum (FBS). All cells were incubated within a humidified incubator at 37°C in 5% CO₂.

Transfection reagents tested include Lipofectamine RNAiMAX (“LF”, Life Technologies) and DharmaFECT 1, 2, 3, and 4 (“DF1, DF2, DF3, DF4”, GE Life Sciences). siGLO Green Transfection Indicator (GE Life Sciences), a FAM-labeled oligonucleotide, was used as a marker for successful siRNA transfection. Oligonucleotides and transfection reagents were diluted in Opti-MEM reduced-serum medium (Life Technologies). 24 hours following transfection, Draq7 (Beckman Coulter) was added.
to cells. After 30 min, media was replaced with FluoroBrite DMEM (Life Technologies) with 10% FBS to reduce background fluorescence in imaging experiments.

Automation: A Biomek FX® Workstation with a 96-channel head and Span-8 pipettors (Figure 1A) was utilized for all liquid transfer steps. The enhanced selective tip (EST) pipetting feature of the 96-channel head, with which any pattern of tips can be used (Figure 1B), enabled the factorial combination of reagent additions across rows and columns. The flexible deck configuration of the Biomek FX® allows for custom integration of additional devices (i.e. incubators, cell viability analyzers) to enable complete automation of cellular workflows. A sterile environment for liquid transfers and cell handling was maintained through the use of a HEPA-filtered enclosure.

Imaging Cytometry: The SpectraMax MiniMax cytometer was used to image cells at 4x magnification in brightfield, 541 nm, and 713 nm emission wavelengths. Cells were analyzed using SoftMax Pro 6.4.2 software. Cells in brightfield images were counted using StainFree Cell Detection Technology with the available CellsD setting, while 541 nm positive cells and 713 nm positive nuclei were identified based on size and intensity threshold. Total cells, transfected cells (siGLO positive), and dead cells (Draq7 positive) were counted from images in these wavelengths respectively. Positively stained cells were compared to total cell counts to determine transfection efficiency and toxicity for each condition. Brightfield cell counts were also compared to negative control wells that lacked transfection reagents.

Automated Transfection Optimization

To optimize cell transfection, one must find the ideal concentration of nucleic acids, transfection reagents, and cell numbers that result in high percentages of transfected cells and low toxicity. This creates a factorial experiment which quickly becomes unwieldy and highly challenging to perform manually, particularly since replicate wells are needed to ensure consistent results. In addition, the cost of these reagents can be significant, particularly when used in large sample numbers. These cost and throughput demands can be mitigated by using higher density plate formats with smaller well volumes and therefore we performed the optimization experiments in 384-well plates.

Reverse transfection was chosen as the method for optimization. This method, which plates cells onto the transfection reagents, can achieve results within 24 hours rather than adding 24 additional hours for plating cells prior to traditional forward transfection. It is also more amenable to high-throughput applications like screening siRNA libraries. The method entails separately diluting the oligonucleotides and transfection reagents in Opti-MEM and then combining these diluted reagents. After 15 minutes, these reagents are plated onto tissue culture plates, followed by cell addition and incubation.

The automated method for optimizing this procedure is shown in Figure 2A. Briefly, 6 tubes of transfection reagents (including a negative control) and 2 tubes of siGLO (1 µM and 500 nM stock solutions) were placed on the deck of the Biomek FX® Workstation. The transfection reagents were serially diluted in Opti-MEM down 4 rows of a 96-well plate and replicate stamped into the bottom 4 rows. 4 wells of each siGLO

Figure 1. Automated cell culture and analysis. A) A Biomek FX® with a 96-channel head and Span-8 pipettors inside a HEPA-filtered enclosure was used to automate sterile cell transfection and reagent additions. B) The enhanced selective tip pipetting feature of the 96-channel head was utilized to create reagent and cell dilutions across rows and columns. C) The SpectraMax MiniMax cytometer was used for analysis of cellular transfection and cytotoxicity.
concentration were stamped across the remaining 6 columns of the plate. The 48 lipid dilution wells were then combined with the 48 siGLO wells, incubated, and stamped into wells of a 384-well black-walled clear bottom tissue culture plate. During the incubation, trypsinized cell suspensions were manually prepared and added to the deck. Two volumes of cells and media were added to alternating rows of the 384-well plate for total cell counts of 7,500 or 15,000 per well. This resulted in quadruplicate wells of each condition (Figure 2B). The total time required for this automated plating was 32 minutes, including incubation times.

Cells were incubated for 24 hours, and no contamination was detected in the absence of antibiotics in the media, indicating the HEPA enclosure and sterile tips were sufficient to maintain cell sterility. To minimize reagent waste during cytotoxicity staining, the EST pipetting was used to transfer a 1:200 dilution of Draq7 from a single column of a round bottom plate to each well of the 384-well culture plate (1:2000 final dilution). After 30 minutes, the 96-channel head was used to replace fluorescent reagent-containing media with FluoroBrite medium to reduce background fluorescence.

### Analysis of Transfection Efficiency and Cell Toxicity

The factorial transfection combinations were tested on three different cell lines – a colorectal carcinoma line (HCT116), a pancreatic epithelioid carcinoma line (PANC-1) and a renal cell adenocarcinoma line (ACHN). The SpectraMax MiniMax cytomter was used to image total cells (brightfield), transfected cells (541 nm), and dead cells (713 nm). Figure 3 shows the images from a single well of PANC-1 cells 24 hours after transfection following Draq7 incubation and media removal.

**Figure 2.** Workflow for automated transfection optimization. A) Liquid handling steps to generate factorial combinations of transfection reagents and siGLO oligonucleotides. Step 1 - transfection reagents (dark orange), Opti-MEM (light orange), and siGLO concentrations (purple) were added to a 96-well plate. Step 2 - transfection reagents were serially diluted across 4 rows and replicate stamped. siGLO was replicate stamped across 5 additional columns. Step 3 - the 48 lipid dilution wells were then combined with the 48 siGLO wells. B) The 48 conditions were replicate stamped into a 384-well plate and cells were added at two concentrations (7,500 or 15,000 cells/well). The representative plate map illustrates the quadruplicate values of 15,000 cells transfected with 100 nM siGLO in the presence of 0.8 µL DharmaFECT 1.
Figure 3. Measuring transfection efficiency and cytotoxicity. PANC-1 cells were transfected with FAM-labeled siRNA oligonucleotides (siGLO) for 24 hours, stained with Draq7 to identify cytotoxic cells, and imaged with the SpectraMax MiniMax cytometer. A) Brightfield image utilized for total cell counts. B) 541 nm image utilized for transfected cell counts. C) 713 nm image utilized for dead cell count. D) Overlay of all three images.

Table 1 summarizes the criteria used to identify the optimal transfection condition(s) for each cell line. Briefly, the optimal conditions were those that maximized the percentage of transfected cells while minimizing cell toxicity. In addition, total cell counts were compared to wells with no lipids added to determine if growth was slowed and/or dead cells were lost during the media replacement step. Finally, the coefficient of variation was calculated for quadruplicate wells to determine the consistency of transfection efficiency for all conditions. To qualify for optimal conditions, wells must illustrate less than 5% toxicity and less than 10% cell loss with transfection efficiency variability at or below 10% CV. If there were multiple conditions that gave equivalent results, then preference was given to conditions that used the least reagents as a way of minimizing costs.

Figure 4 shows the average values of quadruplicate wells for each condition for the three different cell lines. Dark green wells show the wells with >30% transfected cells while red and blue wells have toxicity or cell loss above the acceptable thresholds. The light green wells identify the conditions that gave consistent transfection, as indicated by low %CV values. Optimal conditions are outlined with boxes to identify the lipids and concentrations, siRNA concentration, and starting cell number that maximized transfection efficiency without resulting in significant induction of cytotoxicity or cell loss.

The summary of optimized conditions for the three cell lines can be found in Table 2. Briefly, HCT116 and PANC-1 cells showed robust transfection efficiency of roughly 60%. This was achieved with three different lipid reagents (DharmaFECT 1, 2, and 4) indicating these cells have relatively broad lipid compatibility. In contrast the ACHN cells proved more refractive to transfection. The maximal efficiency achieved without significant toxicity by any of the 5 reagents was 32%, and only one condition was able to achieve >30% efficiency. Additional optimization (i.e. incremental changes from the current optimal settings) may improve this percentage but it is unlikely that the 60% transfection level can be achieved, illustrating the significant potential differences in different cell lines. Toxicity effects (Draq7 positive and/or cell loss) were predominantly driven by the lipid concentration, with 0.8 µL causing toxicity in all three cell lines, however lower siRNA concentrations reduced this effect for some lipids. As expected, variation (%CV) was lowest in the highest transfection conditions. However, some highly transfected conditions still showed significant variability, thereby illustrating the importance of identifying those conditions that give the highest degree of consistency.

### Discussion

Here we have described the automation of a factorial experiment to determine the ideal combination of cell number, siRNA concentration, and transfection reagent and volume to maximize transfection efficiency while minimizing cell toxicity. By miniaturizing the assay to a 384-well plate format, reagent usage was minimized and replicate wells were accommodated so that transfection consistency could be tested on a single plate. We also illustrated broad applicability for this automated approach by utilizing it to rapidly determine optimal transfection conditions for three different cell lines.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Calculation</th>
<th>Goal</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Transfected</td>
<td>541 nm count/brightfield count</td>
<td>Highest</td>
</tr>
<tr>
<td>% Dead</td>
<td>713 nm count/brightfield count</td>
<td>&lt; 5%</td>
</tr>
<tr>
<td>% Control</td>
<td>Brightfield count/avg. control brightfield count</td>
<td>&gt; 90%</td>
</tr>
<tr>
<td>Transfection consistency</td>
<td>Replicates : 541 nm std. dev./541 nm avg. value</td>
<td>Replicate CV ≤ 10%</td>
</tr>
<tr>
<td>When equivalent</td>
<td></td>
<td>Use less reagents</td>
</tr>
</tbody>
</table>
The fluorescent siRNA molecules used in these experiments is just one method of quantifying transfection efficiency. We saw that in some cases, the higher siRNA concentration did not increase the percentage of transfected cells. However, the knockdown effect of siRNAs targeted against native mRNAs may vary with concentration, and hence subsequent studies such as measuring residual gene expression might be required. Another test would be to measure biological significance, such as assaying cell viability following transfection with siRNAs targeting a gene essential for cell survival. Low cell viability in wells containing non-targeting siRNA would still identify cytotoxic transfection conditions, while cell death that is specific to the targeted siRNA would measure on-target effects. The conditions that give the largest spread between specific and non-specific toxicity would be the optimal transfection condition.

Figure 4. Identifying the optimal transfection conditions for three different cell lines. The values represent averages of quadruplicate wells for each transfection condition for A) PANC-1, B) ACHN, and C) HCT116 cells. Combined variables include transfection reagent (LF, DF1, DF2, DF3, DF4, and Con (no reagent)) and volume (0.4, 0.8 µL). siRNA concentration (50 – 100 nM), and cell number (7,500 – 15,000). Wells identified by shading include those above 30% transfected cells (dark red), those above 5% transfected cell death (red), those with less than 90% of the total cell counts (dark blue) and those with transfection CV ≤ 10% (light green). Optimal conditions with maximal transfection efficiency and low variability along with minimal cytotoxicity and cell loss are indicated by black outlines and summarized in Table 2.

Table 2. Optimal transfection conditions and results.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Lipid and volume</th>
<th>siRNA conc.</th>
<th>Cells plated</th>
<th>% TF (541/58)</th>
<th>% Dead (713/58)</th>
<th>% Control (BF/BF)</th>
<th>TF %CV</th>
<th>Equivalent Alternatives</th>
</tr>
</thead>
<tbody>
<tr>
<td>PANC1</td>
<td>0.4 µL DF4</td>
<td>100 nM</td>
<td>7,500</td>
<td>57.4%</td>
<td>2.2%</td>
<td>99.0%</td>
<td>45.3%</td>
<td>0.4 µL DF1, 0.4 µL DF2</td>
</tr>
<tr>
<td>ACHN</td>
<td>0.8 µL DF1</td>
<td>50 nM</td>
<td>15,000</td>
<td>31.6%</td>
<td>1.5%</td>
<td>107.4%</td>
<td>10.0%</td>
<td>0.4 µL DF1, 0.4 µL DF2</td>
</tr>
<tr>
<td>HCT116</td>
<td>0.4 µL DF4</td>
<td>100 nM</td>
<td>7,500</td>
<td>62.8%</td>
<td>1.6%</td>
<td>102%</td>
<td>5.8%</td>
<td>0.4 µL DF1, 0.4 µL DF2</td>
</tr>
</tbody>
</table>
This automated workflow could also be easily adapted to optimize plasmid transfections. The lipid reagents used would likely change but the design of experiment (DOE) would take a similar approach. Transient transfection of a GFP expression plasmid could be measured with the SpectraMax MiniMax cytometer and the optimal conditions could then be utilized to initiate a stable transfection line. In addition, the identification and expansion of strongly expressing clones could be automated by integrating the SpectraMax MiniMax cytometer on a cellular system similar to the one described here.

Scientists are always seeking ways to accelerate the rate of discoveries and technologies and methods than can hasten experimental design and optimization can be of great value. The optimization experiments shown here would be highly challenging and time consuming to execute manually, and therefore, researchers might bypass this level of optimization entirely. By performing the factorial studies with automation, optimized conditions can be identified that may have been missed through sequential testing of single variables. The result is likely weeks saved during optimization and/or improved transfection efficiency over non-optimized conditions.

Authors
Michael Kowalski, Staff Applications Scientist, Beckman Coulter Life Sciences, Indianapolis, IN
Hamidah Sultan, Field Application Scientist, Molecular Devices, Sunnyvale, CA
Kristin Prasauckas, Manager, Field Applications Scientist, Molecular Devices, Sunnyvale, CA
Tara Jones-Roe, Marketing Manager, Beckman Coulter Life Sciences, Indianapolis, IN