# Miniaturizing RNAi Assays: Acoustic Droplet Ejection Enables Efficient and Reproducible Transfer of siRNA Molecules at Low Concentrations

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## Introduction

To facilitate large-scale, high-throughput functional genomics studies using RNAi, we have developed acoustic droplet ejection (ADE) technology to meet the current demands of a successful siRNA library-based screen. High-throughput applications require reliable and reproducible transfections to be performed in high-density well plate formats. The success of an siRNA experiment relies on the effective delivery of the siRNA molecule. Here we have compared the performance of the ADE technology for standard transfection, reverse transfection and Accell (Thermo Scientific) siRNA molecules against more traditional liquid handling methods.

## Acoustic transfer in liposome-mediated transfection

### Example 1: siGLO® siRNA

To compare transfection efficiencies of cell assays prepared using ADE vs. manual pipette dispensing, we used siGLO fluorescent transfection indicators in liposome-mediated transfection assays. siGLO transfection indicators localize to the nucleus and are often used to determine optimal siRNA transfection conditions, or to monitor relative efficiency of delivery when co-transfected with siRNA. In this experiment we compare the transfection efficiencies of siGLO transfection indictor when it is transferred manually by pipette or by ADE technology using the Echo® 555 liquid handler (Labcyte Inc.).

**Methods**

For the ADE approach the Echo 555 transferred volumes between 10nL and 1000nL of siGLO and DharmaFECT3 (Thermo Scientific) transfection reagent into a 96-well flat-bottomed microplate. The plate was then backfilled with 40µL of serum-free RPMI medium. For the manual pipette approach the appropriate dilutions of siGLO and transfection reagent were prepared in a volume of 20µL. Each concentration of siRNA and transfection reagent was performed in triplicate for both the ADE and manual approaches. The siRNA and transfection reagent was incubated at room temperature for 20mins before 160µL KYSE cells ($1.0\times10^3$) were added to the wells. After 24hrs incubation inclusion intensities of the cells were measured using an In Cell Analyzer 1000 (GE Healthcare). Cells transfected with siGLO were also stained with nuclear stain Hoechst 33358 and imaged using fluorescence microscopy (Figure 2A).

**Results**

The data indicated that ADE-transferred siRNAs yield stronger inclusion intensities, and at lower concentrations than manually transferred siRNAs (Figure 2B). Inclusion intensities in ADE-transferred samples are also more directly proportional to the amount of siGLO added than in manually transferred samples.

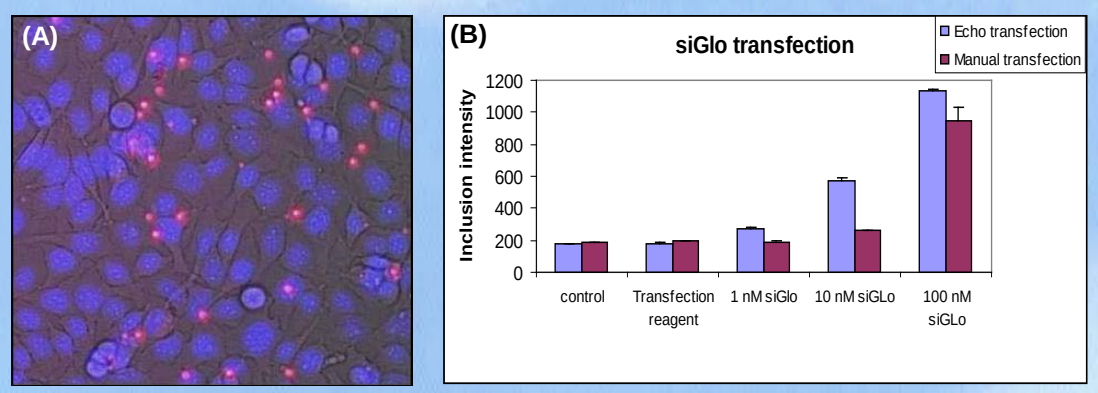


**Figure 2. (A)** Fluorescence image reveals KYSE cells successfully transfected with siGLO (pink) transferred using the Echo 555 liquid handler. **(B)** Inclusion intensities of esphogeal cancer cells transfected using manual and ADE transfer. Error bars indicate standard deviations.

### Example 2: Bcl-xl siRNA

To measure the effectiveness of the knockout of B-cell lymphoma-extra large (Bcl-xl; a mitochondrial transmembrane protein implicated in cancer cell survival) we transferred varying concentrations of Bcl-xl siRNAs and non-homologous scrambled nucleotide controls using both ADE and manual pipette, followed by a cell proliferation (MTT) assay.

**Methods**

For the ADE approach the Echo 555 transferred volumes between 5nL and 500nL of siRNA and DharmaFECT3 transfection reagent into a 96-well flat-bottomed microplate. The plate was then backfilled with 20µL of serum-free RPMI media. For the manual pipette approach the appropriate dilutions of siRNA and transfection reagent were prepared in a volume of 10µL. Each concentration of siRNA and transfection reagent, with and without deoxycholic acid (DHA; induces apoptosis), was assayed in six replicates for both ADE and manual transfer. The siRNA and transfection reagent were incubated at room temperature for 20mins before 80µL SKGT4 cells ($2.0\times10^4$) were added to the wells. The plates were incubated (72hrs, 37°C, 5% $CO_2$), and then half of the wells were treated with 500µM of DCA at 64hrs incubation. MTT assay was performed and absorbance was read at 570 nm.

**Results**

The overall cell proliferation responses were comparable in both transfer methods, exhibiting Bcl-xl knock-down with Bcl-xl siRNAs but not with the scrambled controls, and inhibition of cell proliferation with DHA (Figure 3).

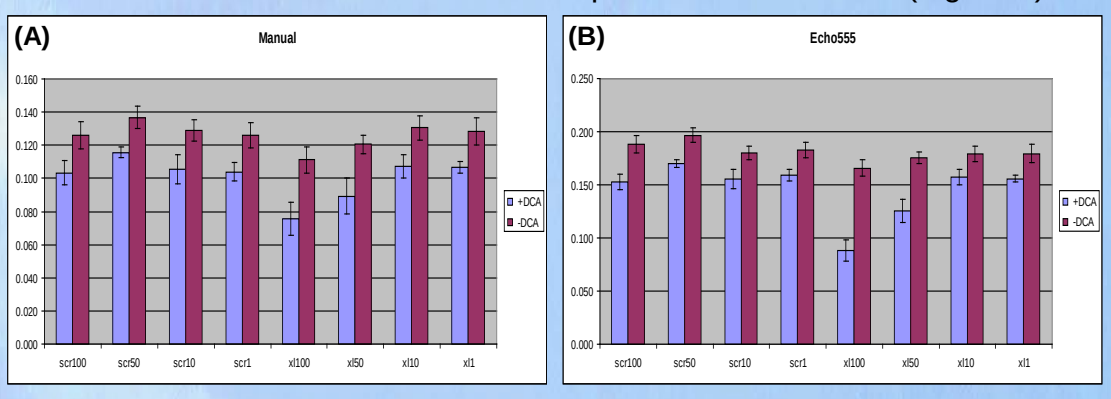


**Figure 3.** Knock-down of Bcl-xl inhibition of SKGT4 cell proliferation by Bcl-xl siRNAs (xl) compared to scrambled (scr) controls, with and without DCA, plotted as optical density ±standard deviation.

A dose-response curve of the knock-down response was calculated as the percent of viable cells treated with DCA relative to untreated controls, normalized against the corresponding scrambled control (Figure 4). The dose-response curve from samples transferred using ADE more accurately reflects the amount of siRNA added than the curve from samples that were transferred manually.

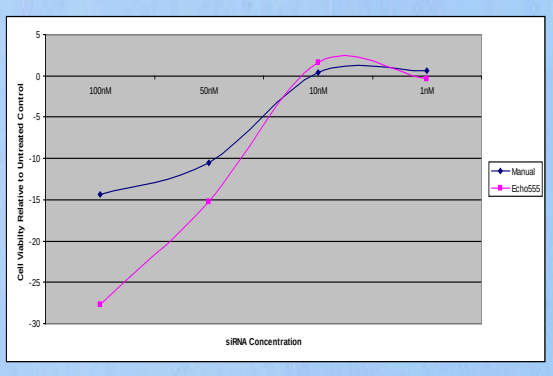


**Figure 4.** Dose-response of Bcl-xl siRNA knock-down of inhibition of SKGT4 cell proliferation, plotted as cell viability relative to the controls.

## Acoustic transfer in lipid-free transfection

To evaluate the performance of ADE for lipid-free transfection, we used Accell siRNAs (Thermo Scientific), which are chemically modified to promote cellular uptake, eliminating the need for conventional lipid transfection reagents.

**Methods**

Accell siRNA plates were reconstituted with 1x siRNA buffer. siRNA was delivered into daughter plates (pre-coated with goat anti-mouse IgG and anti-LFA-1 to enable cell migration) using either a multichannel pipette (Matrix Impact) or the Echo 555 liquid handler. $2.5\times10^3$ peripheral blood T-lymphocytes in Accell siRNA delivery medium were added to each well. The plates were incubated for 72hrs at 37°C, 5% $CO_2$. The cells were fixed, permeabilized and stained with Hoechst 33358 and Phalloidin TRITC. Acquisition and analysis was performed on the In Cell Analyzer 1000. Data was normalized and scored using HITs, a modified version of CELL HTS software created at Trinity College (Boutros et al. *Science* **303**:832, 2004).

**Results**

Five morphological factors were scored using HITs (Figure 5). Cell 1/Form Factor: a measure of roundness which incorporates cell area and perimeter complexity. Cell Area: the two-dimensional surface area enclosed within a boundary. Nuclear Displacement: the position of the nucleus relative to the centroid of the cell. Cell Gyration Radius: the spread of a cell, defined as the square root of the mean squared distance between the cell periphery and its center of gravity. Cell Elongation: length versus breadth of the cell. Both ADE and manual transfer methods successfully identified siRNAs that target proteins that are known to influence T-cell migration.

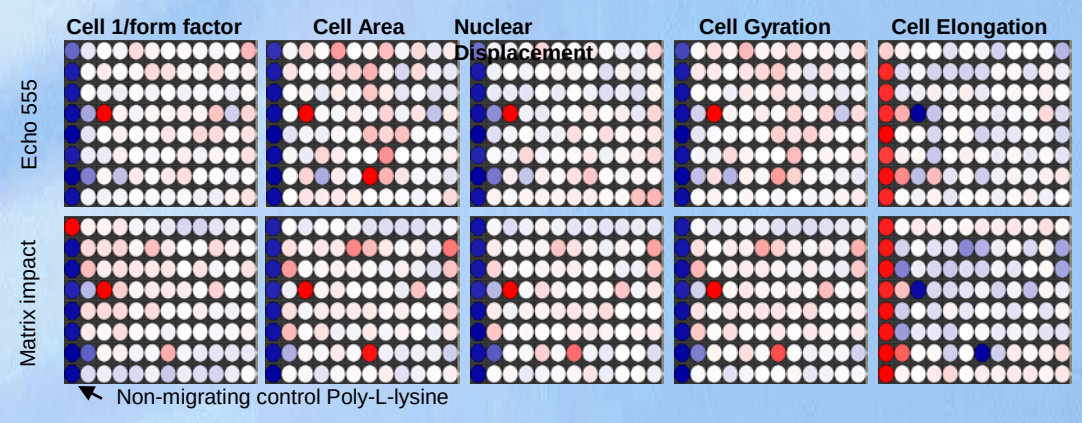


**Figure 5.** Comparison of the Echo 555 and manually delivered siRNAs over five cell morphology factors. Red donates induction and blue denotes repression.

## Conclusions

siRNAs to Bcl-xl have been successfully transferred using ADE technology with no detrimental effects to the siRNA. In lipid-free transfection, ADE successfully identified siRNAs that were known to influence T-cell migration, comparable to manual pipette transfer. In lipid-mediated transfection, ADE transfer yielded more accurate inclusion intensities and dose-response curves than manual transfer. This is possibly due to more effective transfer of the transfection reagent and not the siRNA itself (data not shown).

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