How to Perform High Throughput siRNA Transfections

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Introduction

RNA Interference
In the field of gene silencing, initial work was carried out to study the RNAi pathway in model organisms such as the nematode worm *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster*. In this work, double stranded RNA (dsRNA) constructs of ≈500 base pairs were used for the inhibition of gene expression. However, in mammalian cells (e.g. HeLa), shorter RNA molecules of 21-28 base pairs in length are used because long dsRNA molecules induce the mammalian interferon response[1]. These molecules are termed small interfering RNA (siRNA). The delivery methods of siRNA into cells are varied, electroporation, microinjection, sonofection, etc. One effective way is transfection through lipofection. This method is believed to be especially effective in the cell growth phase. This is achieved by forming a transfection complex of siRNA and a cationic lipid-based transfection reagent which is then incubated with cells to deliver the siRNA molecule into the cytoplasm.

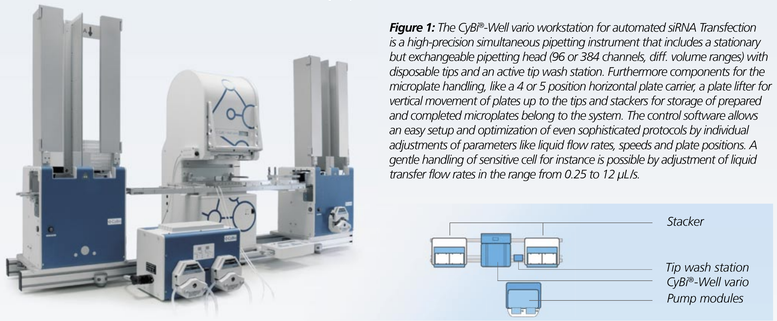

Figure 1: *The CyBi®-Well vario workstation for automated siRNA Transfection is a high-precision simultaneous pipetting instrument that includes a stationary but exchangeable pipetting head (96 or 384 channels, diff. volume ranges) with disposable tips and an active tip wash station. Furthermore components for the microplate handling, like a 4 or 5 position horizontal plate carrier, a plate lifter for vertical movement of plates up to the tips and stackers for storage of prepared and completed microplates belong to the system. The control software allows an easy setup and optimization of even sophisticated protocols by individual adjustments of parameters like liquid flow rates, speeds and plate positions. A gentle handling of sensitive cell for instance is possible by adjustment of liquid transfer flow rates in the range from 0.25 to 12 µL/s.*

Automated Transfection Set-up
The automation of high throughput siRNA transfection requires a multi-channel liquid handling system. In addition to the transfection itself the pipetting system should also be able to perform reformatting/duplication of siRNA libraries and assay plates, the preparation of transfection media, culture media exchange and cell handling. Therefore the important features of the pipettor are the minimization of cross-contamination within siRNA libraries and assay plates, reliability and ease of operation.
In addition, a pipetting instrument which offers the possibility to re-use tips with an effective tip wash station is of particular import. Reduction of cross-contamination is determined by various factors such as: the settings of liquid handling parameters (aspiration/dispense speeds, heights, airgaps, etc.), the wash process itself (number of washing steps, duration, etc.), the type of liquids involved (water, organic solvents or detergents) as well as the physio-chemical nature of the dissolved sample molecules (figure 1).
In former work[4] the performance of an active tip wash station was investigated in washing experiments with a fluorescent dye and various solvents as well as different washing parameters. The results indicated after one wash cycle a carry-over of 0.006 % and after two cycles there was virtually no remaining fluorescence observable (minimum detectable carry-over of the experimental system was at 0.003 %). The result that the tip wash station shows an excellent efficiency was the foundation for the application of tip washing in an experimental system which is especially sensitive to cross contamination, namely siRNA transfection. Due to the fact that RNA interference is not directly comparable with a fluorescence measurement, we created an experimental system for the test of a tip washing procedure in an automated siRNA transfection approach.
For the monitoring of the transfection procedure and the gene silencing, siRNA of two reporter genes were used. Eg5 encodes a motor protein that belongs to the kinesin-like protein family and is an essential component of spindle formation and Eg5 is also required to generate and maintain the spindle's bipolar architecture during cell mitosis, therefore reduced Eg5 expression causes mitotic arrest. The overall phenotypic effect of Eg5, 48 hours after transfection is reduced cell proliferation, morphological changes of the nuclei and rounded rather than flat cells[2].

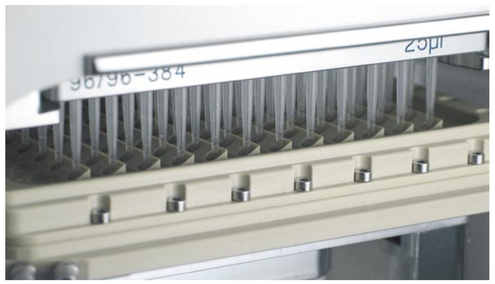

Figure 2: *The active tip wash station utilizes independent clean water chimneys, in this case the 96 tip version, with its separated lower waste reservoir for disposal of liquids ensuring a constant flow of clean untainted wash solutions. It has a source channel and waste flutings for an in/out exchange of solutions for the cleaning of tips. This is driven by two peristaltic pumps.*

The other control gene, GAPDH encodes for an enzyme of glycolysis. The GAPDH siRNA[3] used in this study is known to reduce the expression of GAPDH by 70-95 % in many cell lines including HeLa.

These two positive control siRNA are ideal for developing and optimizing siRNA transfections. Eg5 gives a robust phenotypic effect which can be easily detected with microscopy. GAPDH has a well established mRNA expression knockdown, which can be analyzed by quantitative real-time PCR (qRT-PCR). The negative control Neg1 is an Ambion designed siRNA, which is not targeting any gene and therefore is expected to cause no effect in transfected cells. This is used to account for experimental artefacts from the procedure and/or transfection reagents itself.

We investigated the performance of the tip wash station with a sequence of liquid transfer steps (positive control siRNA complex or negative control), tip washing and reuse of tips for the next liquid transfer (cell culture medium) in a siRNA transfection experiment and subsequent analysis of gene expression in the control samples.

Material

Consumables/Reagents
» Greiner 384 well µClear microplates
» Nunc 384 well Polystyrene microplates
» Hela Cells
» Oligofectamine (Invitrogen)
» Opti-MEM
» 1x Phosphate Buffered Solution (PBS)
» Paraformaldehyde 16% (PFA)
» Eg5 (Kif11) siRNA (50µM Stock)
» GAPDH siRNA (50µM Stock)
» Hoechst (33342) Nuclear Stain
» Invitek' Invisorb RNA extraction kit II
» Applied Biosystems cDNA Reverse Transcription kit
» Quantec Syber Green SensiMix (qRT-PCR reagents)
» DNAse, RNAse free deionised water

Equipment
» CyBi®-Well vario equipped with 96/25µL head
» Active tip wash station for CyBi®-Well vario 96
» CyBi® tips 25µL
» Eppendorf Hand held automatic 8 channel pipette (Various volumes)
» Thermo Multidrop 384
» Applied Biosystems Prism 7900HT
» Molecular Devices Discovery 1 (Automatic Microscope)
» Metamorph V.6.2 software
» Tecan Power Washer 384

Methods

siRNA Transfection
» HeLa cells (2000/well) were seeded on two Greiner µClear 384 microplates in 25 µL of Opti-MEM with a Thermo Multidrop 384 and incubated for 24 hours at 37°C / 5% CO_2.
In parallel the transfection complex was prepared in a 384 well Nunc clear plate by mixing of siRNA with Oligofectamine and using Opti-MEM as a diluent to reach an end concentration of 30nM. Two source plates were generated in the following way:
Source plate type 1 containing GAPDH, Neg1 siRNA + complex

	1	2	3	4	Quadrants	
A	GAPDH	Opti-MEM	Neg1	Opti-MEM	1	2
B	Opti-MEM	Opti-MEM	Opti-MEM	Opti-MEM	3	4

Source plate type 2 containing Eg5, Neg1 siRNA complex

	1	2	3	4	Quadrants	
A	Eg5	Opti-MEM	Neg1	Opti-MEM	1	2
B	Opti-MEM	Opti-MEM	Opti-MEM	Opti-MEM	3	4

» From each source plate, 5 µL of siRNA complex or Opti-MEM was transferred to Greiner plates seeded with HeLa cells by the CyBi®-Well vario with a 96/25µL pipetting head. In detail:
1. siRNA complex in quadrant 1 of the source plate was transferred to quadrant 1 of the cell plate. Pipetting speed was reduced to 3 µL/s (75 rpm) during aspiration and dispensing to avoid disturbing the cells. After transfer the tips were then subjected to a wash of 3 cycles of 25 µL at a pipetting speed of 12 µL/s (300 rpm) in RNase free ddH2O using the CyBio active tip wash station (figure 2).
2. Opti-MEM from quadrant 2 was then transferred to quadrant 2 of the cell plates and washed with the identical settings as previously described. This was repeated for quadrants 3 and 4.

Both source plates were processed with the same method.

» The experimental cell plates were then incubated for a further 48 hours at 37°C / 5% CO_2 and accordingly analyzed by either PCR or microscopy.

qRT-PCR
The cells from the GAPDH transfected plate (source plate 1) were lysed and the RNA was extracted using the Invisorb RNA extraction kit II from Invitek.
Quantitative measurement of mRNA levels by real time RT-PCR was performed using GAPDH primers and a 18s RNA housekeeping primer for normalization.

Functional Analysis
» The cells from the Eg5 transfected plate (source plate 2) were fixed with 10µL of 16% PFA and incubated at room temperature for 30 min. The Plate was then washed with PBS at 3 x 200 µL with the Tecan Power Washer 384 to remove the fixative.
» The cells were stained with Hoechst nuclear stain for 30 min and washed as described above.
» Cell images were acquired using a Discovery 1 automatic microscope at 10x magnification with 9 sites per well using a DAPI wavelength filter.
» The images were then analysed using Metamorph software to count the number of cells in each well.

Results & Conclusions

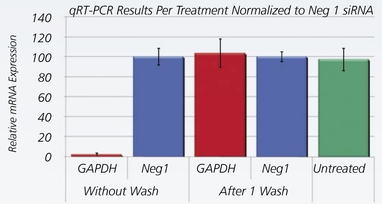

Figure 3: *RT-PCR results showing reduction in mRNA level of GAPDH compared between direct transfer of siRNA complex and the transfer of medium with the same tips after washing.*

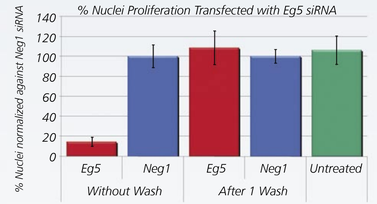

Figure 4: *Image based analysis shows the reduction in cell proliferation in the Eg5 transfected wells. Comparing the difference after 1 wash has been completed and without washing; the second and third wash however was excluded from the results because the first wash showed conclusive results.*

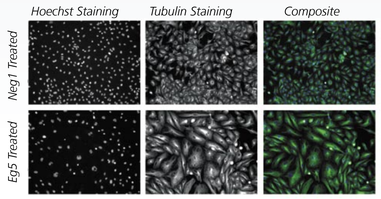

Figure 5: *Representative Images showing the typical phenotypic effect of Eg5 siRNA transfected cells in comparison with Neg1 siRNA transfected cells. The Neg1 treated cells are unchanged in comparison to untreated wild type cells, whereas the Eg5 transfected cells show cell elongation and a reduction in proliferation.*

» GAPDH transfected cells show overall a 90% reduction in mRNA level as measured by qRT-PCR. After 1 wash cycle the relative expression of mRNA for GAPDH is within the range of untransfected samples (figure 3).

» Image based analysis of Eg5 transfected samples shows up to 85 % reduction in cell proliferation. After 1 wash cycle the cell number returns to the range of untransfected samples (figures 4 and 5).

» The experimental results show that 1 wash cycle is sufficient for the removal of any remnants of the siRNA transfection complex. The siRNA effects for both control genes are no longer observed.

» Tip washing utilising specialist equipment (active tip wash station) is a suitable alternative to tip changing to avoid cross contamination in siRNA transfection.

References

[1] Reynolds A, Anderson E, Vermeulen A, Fedorov Y, Robinson K, Leake D, Karpilow J, Marshall W, Khvorova A (2006). "Induction of the interferon response by siRNA is cell type- and duplex length-depend". RNA 12 (6): 988-93
[2] Ambion Cat #4639 Silencer® KIF11 (Eg5) siRNA (Human, Mouse, Rat) Eg5
[3] Ambion Cat #4633/4634 Silencer® GAPDH siRNA (Human) GAPDH
[4] Prüfer H, Busch M (2007). "Performance data demonstrating the efficiency of the tip wash station of the CyBi®-Well family." TechNote CyBio AG, www.cybio-ag.com