QIAGEN News

Integrated, automated system for efficient gene silencing

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Automated procedures for RNA interference (RNAi) would be valuable tools for high-throughput gene silencing experiments, allowing efficient screening for proteins of interest. We used a range of QIAGEN® products on the BioRobot® Gene Expression System to develop an integrated, automated system for gene silencing experiments, which included automated protocols for transfection, RNA purification, and RT-PCR setup.

Now that the human genome has been sequenced, the focus of research is shifting to analyzing the function of the 30,000–40,000 protein-coding genes. RNA interference (RNAi) is a powerful new technique for analyzing gene function. The development of automated protocols for RNAi experiments would allow rapid, large-scale screening for genes of interest.

In this study, we developed protocols to automate all steps of RNAi using the BioRobot Gene Expression System. As a model system, we investigated silencing of the p21-activated kinase (PAK-2) and lamin A/C genes in HeLa and AGS cells. PAK-2 is involved in morphological and cytoskeletal regulation, and lamin A/C encodes a nuclear structural protein. The automated protocols resulted in efficient gene silencing, and provide a new tool for efficient, large-scale testing of the silencing effect of a range of siRNAs.

Materials and methods

HeLa-S3 cells (from human cervical carcinoma) and AGS cells (from human gastric adenocarcinoma) were used for gene silencing experiments. On the day before transfection, 100 µl of cell suspension, containing 2 x 10^4 cells, was plated in each well of a 96-well plate, in RPMI 1640 medium supplemented with 10% FCS.

Cells were transfected on the BioRobot Gene Expression System using a modified protocol for TransMessenger™ Transfection Reagent. Complexes of siRNA and TransMessenger Reagent were resuspended in complete medium containing serum, giving a total volume of 100 µl, and transfection complexes were not removed.

Cells were transfected with siRNAs targeting PAK-2 or lamin A/C (cat. no. 1022050). To eliminate the possibility of nonspecific silencing effects, a negative control was performed using siRNA targeting luciferase (cat. no. 1022070), as luciferase is not expressed in mammalian cells.

RNA was purified from the transfected cells on the second day after transfection using RNeasy® chemistry on the BioRobot Gene Expression System according to the recommended protocol, and including the on-membrane DNase-digestion step.

A new setup for quantitative, real-time RT-PCR analysis was developed using the QuantiTect™ SYBR® Green RT-PCR Kit. Expression of lamin A/C and GAPDH (housekeeping gene, control) was measured, using a standard curve generated by amplifying a dilution series of RNA purified from untransfected HeLa cells. The relative expression levels of GAPDH and lamin A/C in each well were calculated from the standard curve, and the expression of lamin A/C in each well was normalized to the expression of GAPDH. The same method was used to calculate the expression of PAK-2.

Development of automated method

The aim of this study was to develop automated protocols for high-throughput gene

Pipetting Scheme

	1	2	3	4	5	6	7	8	9	10	11	12
A (1.0 µl TM)*		C1			C2			C1			C2	
B (1.5 µl TM)			siRNA 1						siRNA 3			
C (2.0 µl TM)												
D (1.0 µl TM)		C1			C2			C1			C2	
E (1.5 µl TM)			siRNA 2						siRNA 4			
F (2.0 µl TM)												
G		Control					Blank					
H	Standard curve for RT-PCR											

Figure 1 *Cells in each well of a 96-well plate were transfected with different amounts of siRNA and TransMessenger Reagent, as indicated.* ***C1*** *and* ***C2****: Concentration of siRNA;* ***TM****: volume of TransMessenger Reagent.* *******: Volumes of TransMessenger Reagent indicated are examples and should be optimized in individual experiments. An additional four siRNAs were analyzed in a second 96-well plate.*

silencing experiments, using a model system. Results of preliminary experiments showed that use of siRNAs targeted to PAK-2 and lamin A/C resulted in efficient gene silencing (data not shown; see also Figure 2). To monitor the performance of the automated system, each step was performed using manual procedures in parallel with the automated protocols.

The procedure using the BioRobot Gene Expression System enabled seeding from a suspension of adherent cell lines of either four or eight 96-well plates in parallel. Four siRNAs and one control siRNA were analyzed in each 96-well plate (Figure 1). Each plate was set up in duplicate, so that eight siRNAs could be analyzed in four 96-well plates. Duplicate plates allow analysis of gene silencing by different methods (such as analysis of mRNA and protein levels from separate plates).

Optimal conditions for efficient gene silencing vary depending on the gene targeted and the siRNA used, and an automated system must be sufficiently flexible to test a range of transfection parameters. This setup allowed the user to optimize transfection by testing the effect of two amounts of siRNA and three amounts of TransMessenger Reagent on transfection efficiency, performing each transfection in triplicate.

The gene silencing effect was analyzed using automated procedures on the BioRobot Gene Expression System for RNA purification and RT-PCR setup (see "Materials and methods").

Results and discussion

After manual transfection of PAK-2 siRNA into HeLa cells, a preliminary western blotting experiment revealed a strong gene silencing effect (Figure 2). The automated protocol for transfection of siRNA targeting the PAK-2 gene into AGS cells resulted in a greater than fivefold reduction in levels of PAK-2 mRNA, as measured by RT-PCR (Figure 3). Over several experiments, a three- to fivefold reduction in levels of PAK-2 mRNA was observed. Using different amounts of siRNA and TransMessenger Reagent for transfection did not significantly affect the degree of gene silencing.

The automated protocol for transfection of siRNA targeting lamin A/C into HeLa cells resulted in a gene silencing effect of five- to tenfold, as measured by RT-PCR (Figure 4). Using different amounts of siRNA for transfection did not significantly affect the degree of gene silencing. As amounts of TransMessenger Reagent were increased, levels of lamin A/C mRNA decreased slightly, indicating a slightly stronger gene silencing effect. Transfection of AGS cells with lamin A/C siRNA gave very ▶

Suppression of Gene Products

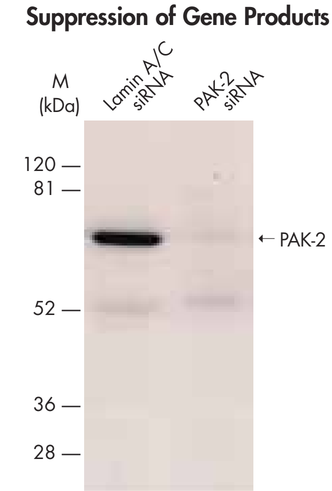

Figure 2 *HeLa-S3 cells cultured in a 12-well plate were transfected with 1.6 µg lamin A/C or PAK-2 siRNA, 3.2 µl Enhancer R, and 6 µl TransMessenger Reagent. Three days after transfection, cell extracts were examined by western blotting with a goat polyclonal antibody directed against PAK-2. All steps were performed manually.* ***M****: markers.*

Efficient Silencing of the PAK-2 Gene

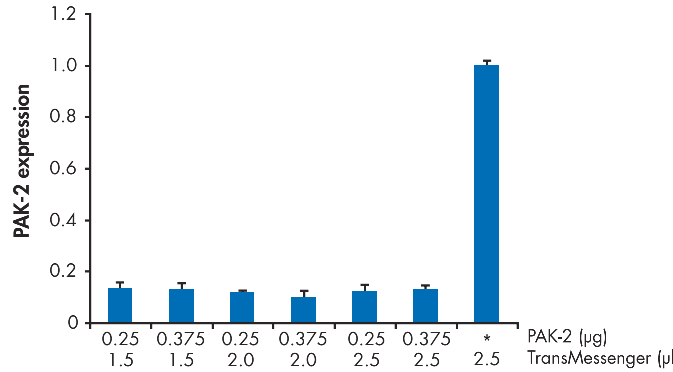

Figure 3 *Relative expression levels of PAK-2 after siRNA transfection into AGS cells, normalized to the expression level of GAPDH, 2 days after transfection. All steps except for cell seeding were performed using automated methods. Amounts of PAK-2 siRNA and TransMessenger Reagent are indicated. PAK-2 siRNA was designed at the Max Planck Institute in Berlin, and was homologous to base pairs 568–586 downstream from the start codon, sequence GAG ACU GCU CCU CCC GUU AdTdT. *: Luciferase (0.375 µg). Values shown are the mean of three readings.*

Efficient Silencing of the Lamin A/C Gene

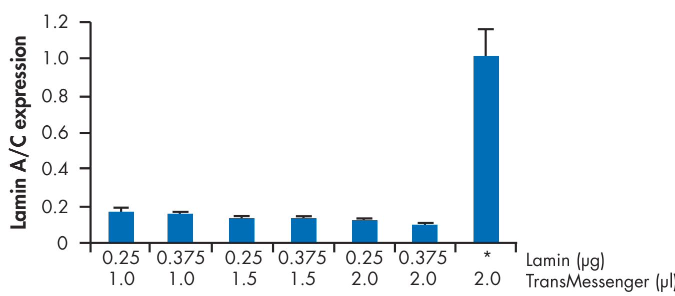

Figure 4 *Relative expression levels of lamin A/C after siRNA transfection into HeLa cells, normalized to the expression level of GAPDH, 2 days after transfection. Lamin A/C siRNA consisted of 21-nucleotide dsRNA duplexes, with symmetric 2-nucleotide 3'-end overhangs targeting lamin A/C at position 608–630 relative to the start codon. Amounts of lamin A/C siRNA and TransMessenger Reagent are indicated. Analysis was performed using automated methods. *: Luciferase (0.375 µg). Values shown are the mean of three readings.*

similar results (data not shown). Optimization of transfection parameters is important when transfecting different siRNAs.

Experiments in which all steps had been performed manually gave comparable results. However the RT-PCR results revealed a slightly higher variation in gene silencing for manually performed and analyzed experiments (data not shown).

Conclusions

- We have developed an integrated, automated system for gene silencing experiments, using a range of QIAGEN products for cell plating, transfection of siRNA, RNA purification, and RT-PCR analysis.
- These procedures reduce the hands-on time required for large-scale gene silencing experiments, allowing efficient high-throughput screening using RNAi.
- The procedures resulted in approximately fivefold silencing of the expression of the lamin A/C gene and a three- to fivefold gene silencing effect for PAK-2, in both HeLa cells and AGS cells. ■

Ordering Information

Product	Contents	Cat. No.
BioRobot Gene Expression System	System includes the BioRobot Gene Expression workstation, QIAsoft™ 4.1 Operating System software and selected protocols, and the Specialist Pack of chemistries, training, and support	9000710
RNeasy 96 BioRobot 8000 Kit (12)	For 12 x 96 total RNA preps on the BioRobot 8000 and the BioRobot Gene Expression workstations: 12 RNeasy 96 Plates, Elution Microtubes CL, Caps, Square-Well Blocks, RNase-Free Reagents and Buffers	967152
TransMessenger Transfection Reagent (0.5 ml)	For 60 transfections in 6-well plates or 80 transfections in 12-well plates	301525

For ordering information for siRNA, see page 30. For details of other products, visit ***www.qiagen.com****.*