A High-Throughput Approach for Rapidly Identifying Knockdown of Gene Expression by RNA Interference

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Abstract

As genome sequencing projects for different organisms are completed, the identification and characterization of the gene function becomes fundamental to understanding the complexity of these systems. This creates a need for comprehensive studies to fill the gap between sequence and function. Many investigative approaches for identifying gene function have been introduced, but RNA interference (RNAi) studies have gained the most prominence due to their potential to enable rapid genome-wide loss-of-function screens in mammalian systems. Quantitation of the mRNA target is typically used to validate successful gene knockdown, and therefore, genome-wide screens require a high throughput approach for isolating and quantifying mRNA level. Standard methods for isolating mRNA can be laborious, time consuming, and not amenable to automation. Therefore, an automated system has been developed for the isolation and subsequent analysis of mRNA that uses Sigma's SpyLine™ Poly A+ Capture kit, a novel system for the rapid isolation of poly A+ mRNA from cultured mammalian cells without centrifugation or vacuum filtration. With SpyLine, mRNA from the cell lysate is selectively bound in a 96-well PCR plate for direct use in quantitative reverse transcriptional PCR (qRT-PCR) analysis. The automated method was used to identify effective RNAi gene knockdown. Results indicate that this approach has both the sensitivity and reproducibility necessary for measuring transcript levels following gene knockdown.

Materials

Unless otherwise indicated, all reagents and materials used in this work were obtained from Sigma-Aldrich (St. Louis, MO). SpyLine Poly A+ Capture Kit (Cat. No. SPY4) was used to isolate mRNA from cultured HeLa cells after the treatments with targeting siRNA, or non-targeting siRNA or without any treatment. SYBR® Green Quantitative RT-PCR Kit (Cat. No. QR0100) was used to detect RT-PCR products of SURF-4 mRNA after captured by SpyLine plate. Quantitative RT-PCR ReadyMix™ (Cat. No. QR0200) was used to quantify mRNA levels of eight genes before or after siRNA treatments. The M-MLV Reverse Transcriptase (Cat. No. M1302) was used for reverse transcription. The primers for amplifying SURF-4 gene were obtained from Sigma-Genosys (Woodlands, TX). The fluorogenic probes were obtained from Applied Biosystems (Foster City, CA). mRNA isolated from human kidney tissue (BioChain Institute Inc., Hayward, CA) was used as the standards for quantitative RT-PCR.

Methods

Cell Culture

HeLa cells were cultured in DMEM medium with 10% fetal bovine serum (FBS) and plated into a 96-well culture plate at 1×10^4 cells/well 24 hours before mRNA preparation using SpyLine Poly A+ Capture Kit or transfection with siRNA.

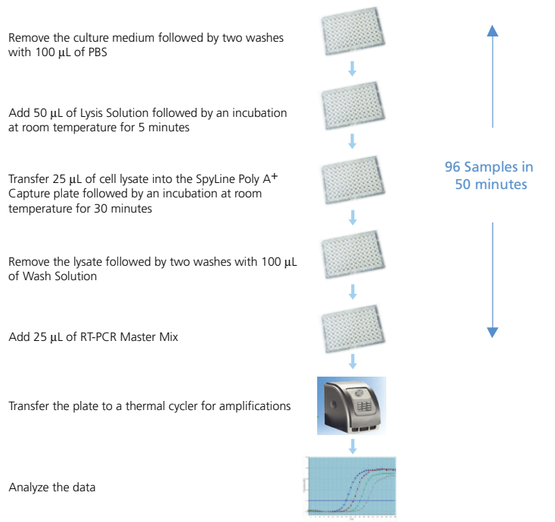
Transfection

The targeting or non-targeting siRNAs were transfected into HeLa cells using commercially available transfection reagent. After 24 hours, the cells were replenished with 100 µL of 10% FBS medium. After 48 hours, mRNA was isolated and quantified.

mRNA Isolation and Quantitation by RT-PCR

The Sciclone ALH 3000 Liquid Handling Workstation was used to isolate mRNA from 96 samples and set up the reactions for quantitative RT-PCR. Reactions included mRNA captured on SpyLine plate or human total RNA controls (2 µL), 2x Quantitative RT-PCR ReadyMix, primers, SYBR® Green dye or fluorogenic probes, Reference Dye, and M-MLV Reverse Transcriptase for a total reaction volume of 25 µL. Quantitative RT-PCR was performed in an ABI PRISM® 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) or Stratagene Mx3000P® (Stratagene, La Jolla, CA).

Automated SpyLine Poly A+ Capture Protocol



Results

Validation of the Automated SpyLine Poly A+ Capture Method

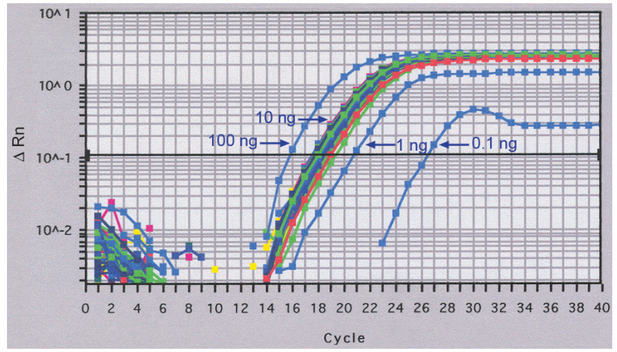


Figure 1. Amplification Plots of Quantitative RT-PCR Analysis of SURF-4 Gene Expression in HeLa Cells. HeLa cells were plated into 96-well culture plate at 1×10^4 cells/well. After 24 hours, mRNA was isolated and the RT-PCR reactions were set up by the automated Sciclone method. Quantitative RT-PCR was analyzed using ABI PRISM® 7700 Sequence Detection System . The SYBR Green dye was used to detect amplified RT-PCR product of SURF-4 gene. All 88 samples showed consistent amplification with a standard deviation of 0.3 Ct. The arrows indicate the amplification plots of the standards with the indicated amount of mRNA added in the RT-PCR reactions.

Application of SpyLine Poly A+ Capture Kit in the Detection of Gene Knockdown by RNA Interference

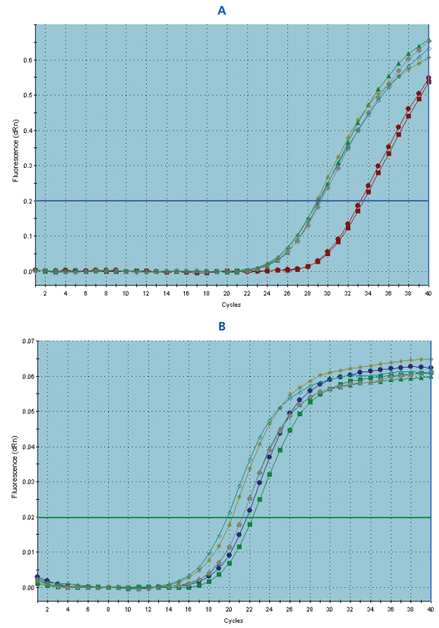


Figure 2B

Figure 2. Amplification Plots of Quantitative RT-PCR Analysis of CHUK Gene Expression in HeLa Cells after siRNA Treatments. HeLa cells were transfected with siRNA targeting CHUK mRNA or with non-targeting siRNA. After 48 hours, mRNA was isolated and the RT-PCR reactions were set up by the automated Sciclone method. Quantitative RT-PCR was analyzed using Stratagene Mx3000P System. The TaqMan probes labeled with different florescent dye for either CHUK (A) or GAPDH (B) were used for the detection. The sample names and the corresponding Ct values of each plot are shown in **Table 1**.

siRNA	PCR Target	Reverse Transcriptase (+/–)	Ct (dRn)		
			1	2	3
Target	CHUK	+	31.22	31.43	31.48
Target	CHUK	–	No Ct		
Target	GAPDH*	+	21.69	22.25	22.8
Target	GAPDH*	–	No Ct		
Non-target	CHUK	+	26.67	27.25	27.3
Non-target	CHUK	–	No Ct		
Non-target	GAPDH*	+	19.97	21.23	21.24
Non-target	GAPDH*	–	No Ct		
None	CHUK	+	26.99	26.87	26.61
None	CHUK	–	No Ct		
None	GAPDH*	+	20.25	19.76	19.62
None	GAPDH*	–	No Ct		

*The mRNA levels of Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH) in each sample were determined for the normalization of CHUK mRNA levels.

Table 1. Quantitative analysis of gene expression levels of Conserved Helix-loop-helix Ubiquitous Kinase (CHUK) in HeLa cells after siRNA treatments using Stratagene Mx3000P System. Example of raw data used to calculate mRNA knockdown shown in **Figure 3**.

Comparison of % Knockdown of Targeting vs. Non-Targeting siRNA

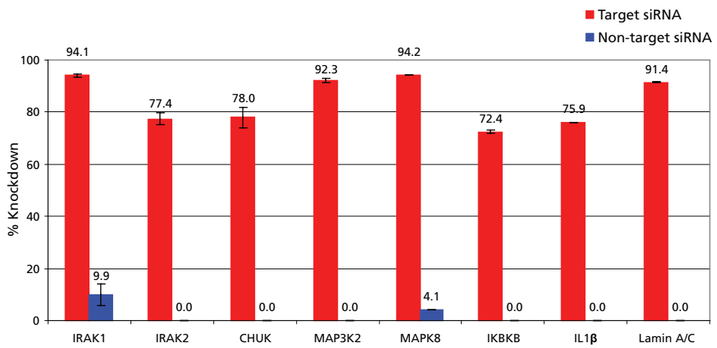


Figure 3. Knockdown of gene expression levels of eight different genes after the transfection with targeting siRNA in HeLa cells. HeLa cells were transfected with targeting or non-targeting siRNA of IRAK1, IRAK2, CHUK, MAP3K2, MAPK8, IKBKB, IL-1β, or Lamin A/C gene. After 48 hours, mRNA was isolated and the RT-PCR reactions were set up by the automated Sciclone method. Quantitative RT-PCR was analyzed using Stratagene Mx3000P System. The percentage of knockdown was calculated for the cells treated with targeting or non-targeting siRNA for each gene relative to the untreated cells after being normalized with internal GAPDH gene expression level using comparative Ct method. An example of the calculation formula can be expressed as $\%\text{knockdown} = 100 - (1/(2^{((Ct_{gene}-Ct_{GAPDH})_{target} - (Ct_{gene}-Ct_{GAPDH})_{untreated})})\bullet100)$. All eight targeting siRNAs showed effective knockdown of gene expression at the transcriptional level. The standard deviations were obtained from three replicates.

Conclusions

- SpyLine Poly A+ Capture Kit offers a simple, rapid, and cost-effective method for isolating mRNA from cultured mammalian cells for direct RT-PCR analysis.
- An automated protocol for SpyLine Poly A+ Capture Kit developed on Sciclone ALH 3000 workstation enables high-throughput mRNA preparations and subsequent analysis. 96 samples can be processed in 50 minutes from mRNA isolation to RT-PCR reaction setup.
- The automated method was applied to identify effective RNAi gene knockdown with both the sensitivity and reproducibility necessary for measuring transcript levels following gene knockdown.

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