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Alexa Fluor labeled siRNA is highly effective for monitoring transfection efficiency

Dong Liang, John Lumpkin, James Qin, Eric Lader, and Subrahmanyam Yerramilli
QIAGEN Sciences, Germantown, MD, USA

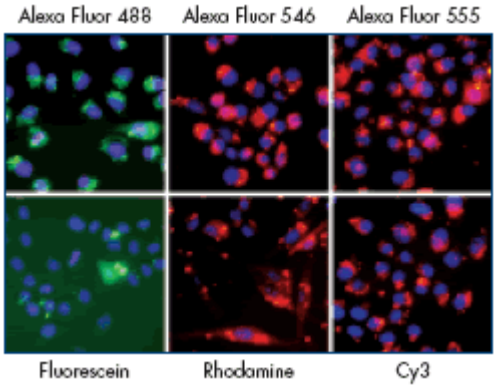
HeLa S3 cells were transfected with siRNA labeled with a range of fluorescent dyes and transfected cells were examined for brightness and duration of fluorescence, and for gene silencing efficiency. Results showed that non-silencing Alexa Fluor labeled negative control siRNA from QIAGEN is highly suitable for RNAi optimization and control experiments, and provides clear advantages over siRNA labeled with traditionally used dyes. HPP (High-Performance Purity) Grade siRNA provided highly efficient gene silencing

Successful RNAi experiments depend on effective delivery of siRNA into cells. Variability and low transfection efficiency are often limiting factors in gene silencing experiments, particularly in difficult-to-transfect cells such as primary neurons or suspension cells. siRNA duplexes labeled with fluorescent dyes are frequently used to monitor transfection efficiency, allowing optimization of experimental conditions. To date, fluorescent dyes such as rhodamine and fluorescein have been widely used to label siRNA for this purpose. However, disadvantages of using these dyes include lack of photostability, insufficient brightness at low siRNA concentrations, short duration of fluorescence, and inability to identify the location of labeled siRNA in cells. Alexa Fluor dyes are, in contrast, more photostable and brighter than traditional fluorescent dyes. In this study, siRNA duplexes targeted against CDC2 and a non-silencing control siRNA were labeled with a range of traditional and Alexa Fluor dyes. The brightness and duration of fluorescence in HeLa S3 cells transfected with the labeled siRNA was compared. The effect of transfection of different concentrations of labeled siRNA on fluorescence was examined and the gene silencing efficiency achieved using labeled siRNA was assessed. Functionality of siRNA duplexes labeled at either the 5' or the 3' end of the sense strand was determined.

Materials and methods

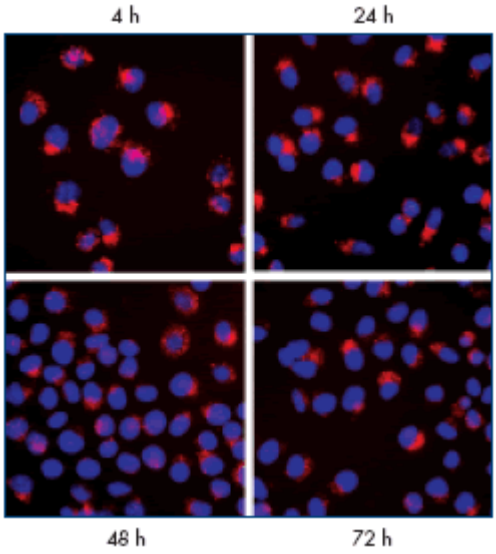
The siRNA target sequences were as follows: non-silencing control siRNA 5'-AAT TCT CCG AAC GTG TCA CGT-3', CDC2 siRNA 5'-AAG GGG TTC CTA GTA CTG CAA-3'. The sense strand of each siRNA was labeled at either the 5' or 3' end with one of the following dyes: fluorescein, tetramethyl rhodamine, Cy3, Cy5, Alexa Fluor 488, Alexa Fluor 546, Alexa Fluor 555, or Alexa Fluor 647. HeLa S3 cells were seeded in 24-well plates at a density of 0.5–1.0 x 105 cells per well. After 24 hours, cells were transfected with siRNA at a final concentration of 100 nM, 10 nM, or 1 nM using RNAiFect Transfection Reagent. After 4, 24, 48, or 72 hours, cells were viewed by fluorescence microscopy. Cell nuclei were stained with Hoechst 33342 before microscopic analysis.
Cells were examined using an Olympus IX71 fluorescence microscope, and the appropriate filter (GFP filter for fluorescein and Alexa Fluor 488; TRITC filter for rhodamine, Cy3, Alexa Fluor 555, and Alexa Fluor 546; DAPI filter for Hoechst 33342). Cell morphology was also examined under white light. After 48 hours, cells were harvested and lysates were prepared. Lysates were separated on 12% SDS-polyacrylamide gels prior to western blot analysis using CDC2-specific monoclonal antibody and anti-mouse secondary antibody. Blots were also probed with tubulin-specific antibody as an internal control. Bands were visualized using chemiluminescence.

Alexa Fluor Labeled siRNA Provides Brightest Fluorescence



Fluorescence microscopy of HeLa S3 cells 24 hours after transfection with 100 nM non-silencing siRNA labeled at the 3' end of the sense strand with different fluorescent dyes.

Long Duration of Alexa Fluor Label Fluorescene in Transfected Cells



Fluorescence microscopy of HeLa S3 cells 4, 24, 48, and 72 hours after transfection with 1 nM non-silencing siRNA labeled at the 3' end of the sense strand with Alexa Fluor 546.

Results

The brightness and duration of the fluorescence of cells 24 hours after transfection with non-silencing siRNA labeled with fluorescein, rhodamine, Cy3, Alexa Fluor 488, Alexa Fluor 546, and Alexa Fluor 555 was observed by fluorescence microscopy. These results showed that Alexa Fluor 546 provided the brightest fluorescence. The fluorescence observed from Alexa Fluor 555, Alexa Fluor 488, and Cy3 was significantly brighter than that of fluorescein and rhodamine (see figure "Alexa Fluor Labeled siRNA Provides Brightest Fluorescence"). Comparison of fluorescence levels of cells 48 hours after transfection with siRNA duplexes labeled at either the 5' end or the 3' end of the sense strand indicated that the end labeled did not affect fluorescence (data not shown). Cell viability and morphology were not affected by transfection of labeled siRNA.

Fluorescence levels were examined 4 hours, 24 hours, 48 hours, and 72 hours after transfection. Alexa Fluor 546 fluorescence was still visible 72 hours after transfection (see figure "Long Duration of Alexa Fluor Label Fluorescene in Transfected Cells"). Further results indicated that Alexa Fluor 546 fluorescence is still detectable up to one week after transfection (data not shown). The results of the time course experiments clearly indicate the increased brightness and duration of fluorescence of Alexa Fluor dyes in comparison to Cy3, fluorescein, and rhodamine (see

table "Percentage Fluorescent Cells Observed after Transfection with siRNA Labeled with a Range of Dyes"). The increased brightness and longer duration of Alexa Fluor Fluorescene allows greater flexibility in RNAi experiments. Experiments were carried out to determine the lowest concentration of labeled siRNA duplexes that provides sufficient cell fluorescence. Labeled siRNA duplexes were transfected at final concentrations of 1 nM, 10 nM, and 100 nM (see table "Percentage Fluorescent Cells Observed after Transfection with siRNA Labeled with a Range of Dyes"). For Alexa Fluor 546 and Alexa Fluor 555 labeled siRNA, fluorescence was visible after 72 hours even when transfected siRNA concentration was as low as 1 nM (see table "Percentage Fluorescent Cells Observed after Transfection with siRNA Labeled with a Range of Dyes" and figure "Long Duration of Alexa Fluor Label Fluorescene in Transfected Cells"). For Alexa Fluor 488 and Cy3 labels, 10 nM siRNA was necessary for visible fluorescence after 72 and 48 hours respectively. Labeled-siRNA concentrations of 100 nM were needed to see fluorescein or rhodamine fluorescence.

The sense strand of the siRNA was labeled in these experiments as this strand is thought not to participate in gene silencing. siRNA duplexes targeted against CDC2 mRNA were labeled on either the 5' end or the 3' end to check whether the end labeled affects siRNA functionality. Western blot analysis was carried out to monitor the gene silencing effect on CDC2. The results show that labeling siRNA at either the 5' or the 3' end does not influence the gene silencing effect (see figure "Labeled siRNA Provides Highly Efficient Gene Silencing"). The results were confirmed by quantitative real-time RT-PCR (data not shown).

Percentage Fluorescent Cells Observed after Transfection with siRNA Labeled with a Range of Dyes

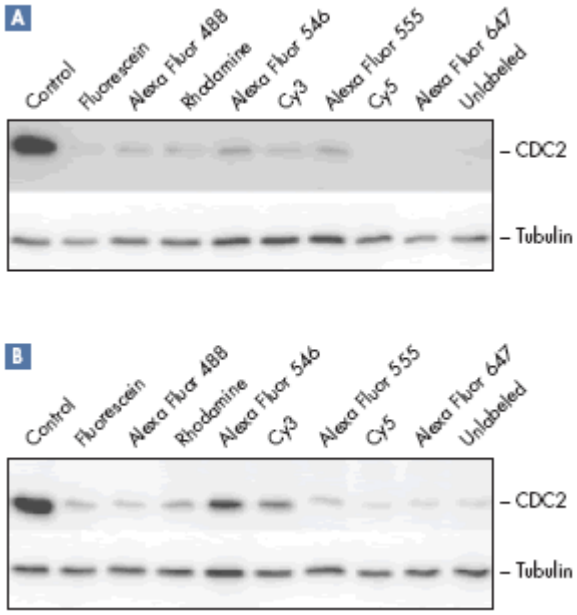
Dye	4 h post-transfection	24 h post-transfection	48 h post-transfection	72 h post-transfection
Alexa Fluor 546, 100 nM	100%	100%	100%	100%
Alexa Fluor 546, 10 nM	100%	100%	100%	100%
Alexa Fluor 546, 1 nM	100%	100%	100%	100%
Alexa Fluor 488, 100 nM	100%	100%	100%	100%
Alexa Fluor 488, 10 nM	100%	100%	100%	50–60%
Alexa Fluor 555, 100 nM	100%	100%	100%	100%
Alexa Fluor 555, 10 nM	100%	100%	100%	100%
Alexa Fluor 555, 1 nM	100%	100%	100%	100%
Cy3, 100 nM	100%	100%	100%	50%
Cy3, 10 nM	100%	100%	100%	0%
Fluorescein, 100 nM	10%	5%	1%	0%
Rhodamine, 100 nM	60–70%	60–70%	40–50%	0%

Conclusions

- Cells transfected with Alexa Fluor labeled siRNA showed brighter fluorescence than those transfected with siRNA labeled with traditionally used dyes.
- Alexa Fluor fluorescence has a long duration in cells, allowing transfection to be easily monitored after 72 hours.
- Fluorescence from Alexa Fluor labeled siRNA is visible after transfection of siRNA concentrations as low as 1 nM.
- Fluorescent dye-labeled siRNA duplexes targeted against CDC2 provided highly efficient gene silencing. Gene knockdown levels were not influenced by whether the sense strand was labeled on the 5' or the 3' end.
- Alexa Fluor labeled siRNA is highly suitable for use in transfection optimization experiments. Low concentrations of Alexa Fluor labeled siRNA can also be used to spike siRNA used in transfection, allowing

transfection and the fate of siRNA in the cell to be monitored.

Labeled siRNA Provides Highly Efficient Gene Silencing



HeLa S3 cells were transfected with siRNA targeted against CDC2. siRNA was unlabeled or labeled at **A** *the 3' end or* **B** *the 5' end of the sense strand. Cells were also transfected with control, non-silencing siRNA. After 48 hours, cells were harvested for western blot analysis, as described in materials and methods.*

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