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NEW PRODUCTS

Targefect-siRNA transfection kit for RNA interference studies!

Background on RNA interference: RNA interference refers to the process of sequence-specific , posttranscriptional gene silencing initiated by doulble–stranded RNA (dsRNA) that is homologous in sequence to the silenced gene. This phenomena was previously recognized in plant and insect cells. More recently , Elbashir et al (Ref.1) have shown for the first time that short dsRNA introduced into mammalian cells can result in sequence-specific inhibition of target mRNA without triggering an interferon response. This finding opens up the possibility of using this technology for analysis of gene function in cultured human cells and also opens new avenues for development of gene-specific therapeutics. . RNA interference leads to the inhibition of protein expression by utilizing sequence-specific, dsRNA-mediated destruction of target messenger RNA (mRNA). The short dsRNAs, referred to as small interfering RNAs (siRNA), act catalytically at sub-molar ratios to cleave greater than 95% of the target mRNA in the cell. Studies in C elegans (ref.4) have shown that injection of ds RNA resulted in much more efficient silencing than injection of either sense or antisense stransds alone.The RNA interference effect can be long lasting (4) and may be detectable after several cell divisions. These properties make siRNA extremely effective at inhibiting target gene expression. And open up the possiblitiy of using RNA interference as a tool for functional genomics.

The Targefect-siRNA transfection kit contains a combination of novel reagents that enables highly efficient transfection of functionally active siRNA with significantly reduced levels of cell damage as compared to cationicliposome based transfection reagents. The transfection protocol is extremely simple.

Highly efficient delivery of fluorescent labeled siRNA and gene silencing using the Targefect-siRNA kit has been demonstrated into a variety of cell types such as, Human umbilical vein endothelial cells and endothelial cell lines, vascular smooth muscle cells, B lymphocytes, HEK 293 cells, lens epithelial cells, Hela, Cos-7, MDA and NIH 3T3 cells without any detectable cell damage. Experiments involving transfection of luciferase –directed siRNA into HEK 293 cells expressing the luciferase gene has resulted in a 85% reduction in luciferase activity within 24 hrs after transfection of the siRNA. Efficient silencing of endogenous genes by transfection of the appropriate siRNA has also been demonstrated in HUVECs, smooth muscle cells, HepG2 cells, MDA cells and several commonly used cell lines. The main advantages of the Targefect-siRNA reagent are it's ability to efficiently deliver siRNA even into difficult to transfect cell types such as HUVECs, lymphocytes and muscle cells with almost complete silencing of endogenous gene expression with a single round of siRNA transfection, the applicability of the reagents for in vivo and ex vivo gene silencing experiments and the ability to obtain sustained geen transfer by transfecting every 4th day. The data presented on the following pages provides suppor tor the versatility of the targefect siRNA kit for gene silencing studies.

References:

1. Elbashir, S.M. et al. (2001) Nature 411:494-498.
2. Sharp, P.A. (2001) Genes and Development 15:485-490.
3. Caplen, N.J. et al (2001) Prot. Natl. Acad. Sci. 98:742-9747.
4. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE and Melto CC (1998) Potent and specific interference by double-stranded RNA in Caenorhabditis elegans. Nature 391: 806-811
5. Sharp PA and Zamore PD (2000) RNA interference. Science ,287:2431-2433.

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Targefect-siRNA transfection kit (catalog#0060) _____ $200.00
One kit is sufficient for 100-200 transfections in 35 mm dishes.

Data On Gene Silencing Using The Targefect siRNA Kit:
Analysis Of Gene Silencing In Human Endothelial Cells By Western Blot Analysis:

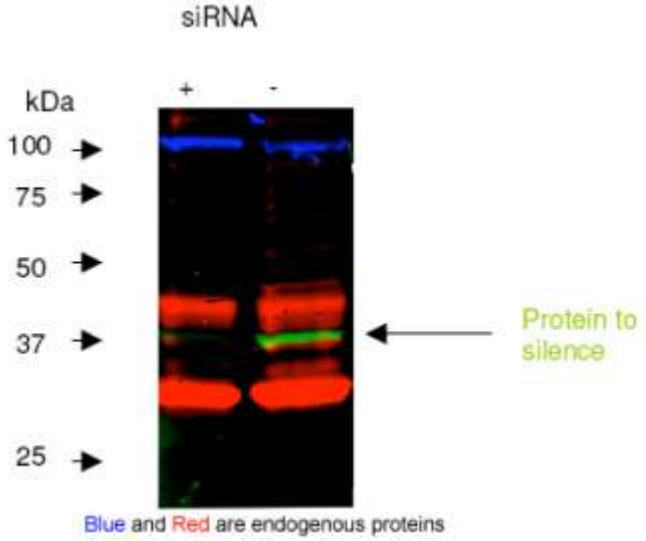

Figure 1: Gene silencing in primary HUVECs: Transfection performed with 75 pmols siRNA complexed with 5 ul soln A and 15 ul of soln. B as per the suggested protocol. Cells were harvested and examined for silencing of endogenous gene expression at 48 hrs post transfection using Western blot analysis (Data courtesy of Dr Eugene Tkachenko, Dartmouth Medical School, USA)

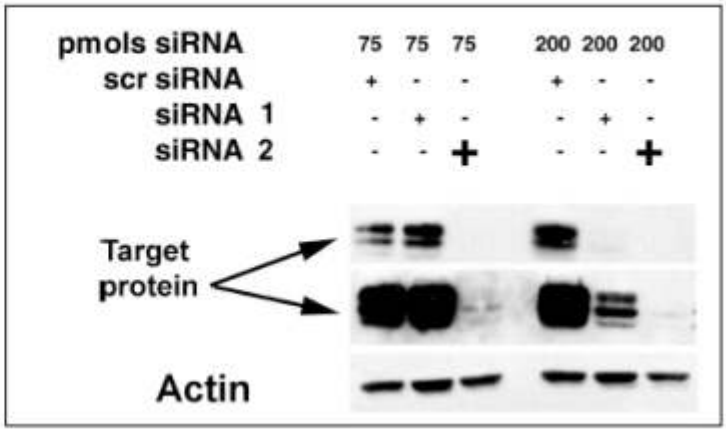

Figure 2: SiRNA-mediated silencing of PV-1, a cell surface glycoprotein in primary HUVECs using the targefect siRNA transfection kit.. (Data courtesy of Dr Radu Stan, University of California at San Diego, La Jolla, California.)

Why Targefect?

- Efficient gene silencing in several cell types.
- Simple transfection protocol
- Sustained gene silencing atleast 4 days with a single round of transfection.
- Extended silencing possible with a second round of transfection. Persistent gene silencing can be maintained for several weeks by transfecting every 3rd. Day.
- Low toxicity
- User friendly optimized protocol
- Suitable for in vitro, ex vivo as well as in vivo gene silencing
- Offers the advantages of a plasmid based or adenoviral-based siRNA expression system without the hassle of constructing recombinant palsmids or recombinant adenovirus

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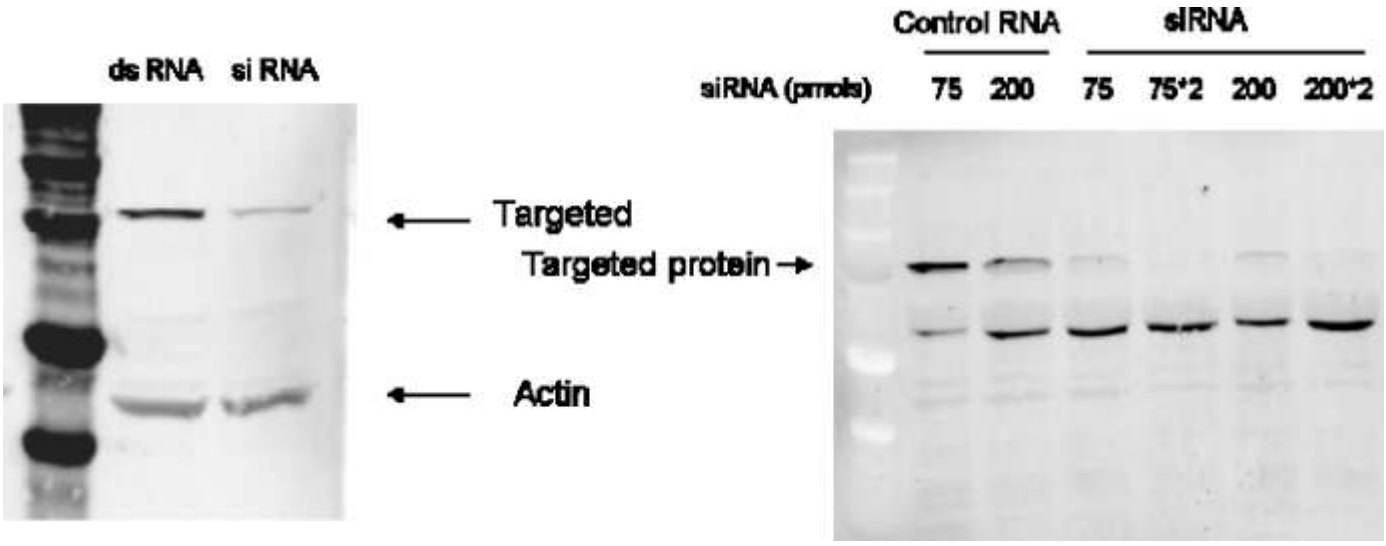
List of Cell Types tested for gene silencing using the targefect siRNA transfection kit:

- Human vascular endothelial cells
- Human microvascular endothelial cells
- Vascular smooth muscle cells
- Primary hepatocytes
- HEK-293 cells
- HepG2 cells
- MDA cells
- Human lens epithelial cells
- Rat lens epithelial cells
- B lymphoma cell lines
- Mouse fibroblasts
- Hela cells
- Cos-7 cells
- A549 cells
- NIH 3T3 cells

Figure 3; SiRNA-mediated gene silencing in rat aortic smooth muscle cells: Silencing of an endogenous gene in primary rat aortic smooth muscel cells using a single round of siRNA (75 pmols per transfection (top panel) . The bottom panel shows the same experiment repeated with using both 75 pmols ans 200 pmols siRNA per transfection (cells in 35 mm dish). Lanes with an asterik after 75 or 200pmols siRNA denote experiments in which cells were transfected a second time with siRNA approximately 24 hrs after the first transfection (to achieve more complete silencing). (Data courtesy of Dr Masahiro Murakami, Dartmouth School of Medicine, Lebanon, NH)



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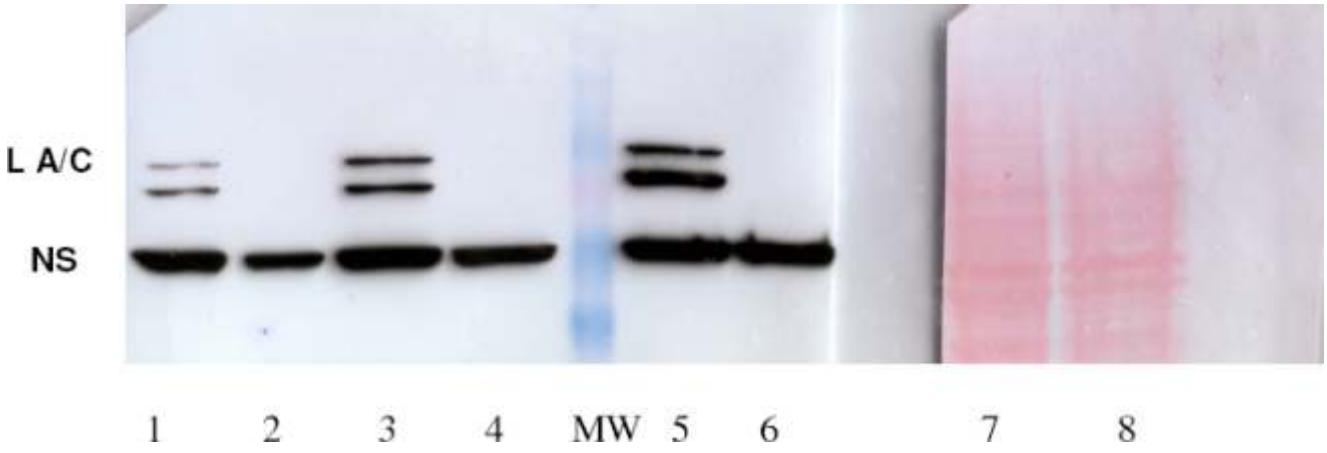

Figure 4: Silencing of lamin expression in MDA 231 cells: The experiment was performed using 75 pmols siRNA , 5 ul of solution A and 10 ul of solution B. Data courtesy of Dr N Vigneswaran, MD Anderson Cancer Center, Hoiuston, TX.

Lanes 1, 3, 5: Mock transfections (1: 5 µl; 2: 10 µl; 3: 15 µl of cellular extract)

Lanes 2, 4, 6: anti –Lamin siRNA transfection. (2: 5 µl; 4: 10 µl; 6: 15 µl of cellular extract)

Lanes 7: Mock transfection- Ponceau S stain – 20 µl extract protein

Lane 8: anti-Lamin siRNA transfection – Ponceau S stain – 20 µl extract protein LA/C: Laminin A/C

NS: Non-specific band – cross react with anti-Lamin A/C antibody (Santa Cruz Bitotech)

Lamin siRNA (DHARMACON RESEARCH, Cat # P-002019)

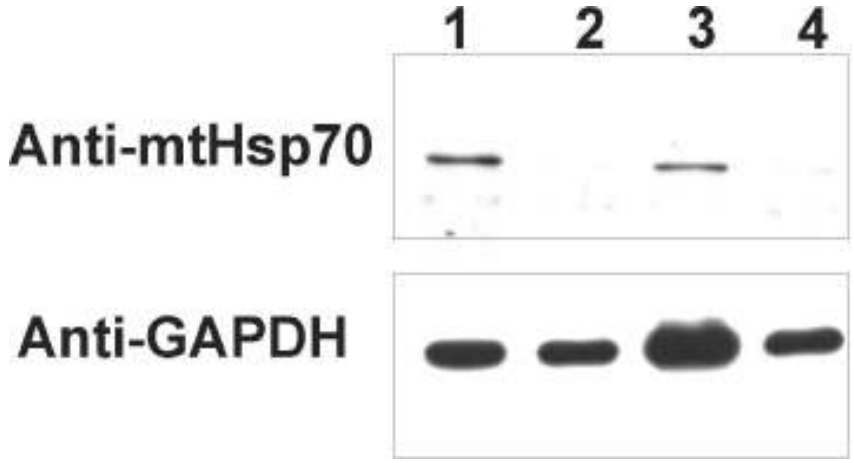

Figure 5: Gene silencing in a mouse fibroblast cell line : (Data courtesy of Dr Julian Leibowitz, Texam A and M University, College Station , Texas.) The data presented shows a western blot of an experiment using 2 siRNAs targeted to the mitochondrial protein mitochondrial hsp70. Samples were harvested 3 days after transfection with siRNAs and analyzed by western blot with anti-mtHsp70 and anti-GAPDH. The western blot of this experiment is shown below. Lane 1 represents lysate from cells transfected with 200 pmoles of an siRNA directed against luciferase, lane 2 lysate from cells transfected with 100 pmoles each siRNAs 1 and 2; lanes 3 and 4 represent lysates from cells transfected with 200 pmoles of siRNA 1 or siRNA 2, respectively. Quantitation by densitometry indicated that the mixture of siRNAs 1 and 2 decreased expression to about 4% of the level seen with the control siRNA directed against luciferase. Transfection with siRNA 1 and 2 decreased levels to 70% and 8% of the culture transfected with the luciferase siRNA, respectively.

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Data on siRNA delivery into human lens epithelial cells and rat lenses: (Data courtesy of Dr. Naseem Ansari and Dr Sanjeev Choudhury, University of Texas Medical Branch, Galveston, TX.)

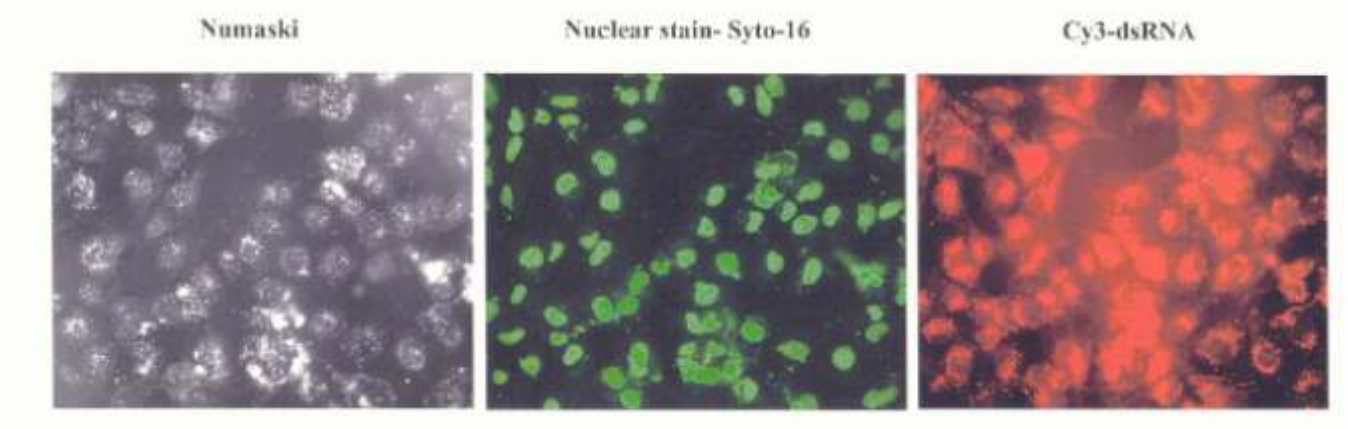

Fig.14. HLEC transfection with siRNA(Cy3-dsRNA): 0.1x106 cells were seeded overnight and transfected with Cy3 labeled 23 bp dsRNA using Targefect siRNA Kit (Targeting systems, Santee, CA), for tow hours. Transfecting reagent was replaced with regular culture media containing 10% serum. Cells were counterstained with nuclear stain, Sito-16 (Molecular probes, Eugene, OR) for 30 min and visualized under a Nikon Eclipse 800 epifluorescence microscope equipped with a xenon arc lamp using Plan Flour 40X 0.75 objective. Photographs were taken using a ROPER Scientific CoolSNAP Fx monochrome cooled CCD 12 bit digital camera. Cy-3 dsRNA and nucleus appears red and green, respectively.

Data on siRNA delivery into human lens epithelial cells and rat lenses: (Data courtesy of Dr. Naseem Ansari and Dr Sanjeev Choudhury, University of Texas Medical Branch, Galveston, TX.)

Delivery of ALDH1 siRNA into rat lenses: The lens in culture were transfected with 100 nM siRNA designed specifically against rat ALDH1 for 6 hrs using the targefect siRNA kit. The transfecting media was changed to media 199 and cultured overnight. Transfected lenses were used to study the effect of ALDH1 silencing after 2 hrs of transfection as assessed by ALDH1 expressiona nd activity.

ALDH1 expression: The epithelium of the untransfected and siRNA-transfected rat lenses were removed, homogenized in 10 mM potassium phosphate buffer pH 7, and centrifuged. Twenty ug protein of the soluble fraction from each was subjected to SDS-PAGE and immunoblots developed using anti-ALDH1 polyclonal antibodies. Figure 1 shows more than 505 decrease in ALDH1 expression in the lens epithelium of the siRNA-transfected lenses as compared to untransfected lenses.

ALDH1 activity: ALDH1 activity was assessed by incubating the untransfected and siRNA – transfected lenses with 30 nmoles of 3H-HNE for 1 hr and analyzing it's oxidation to HNA after separation by HPLC, using a gradientconsisting of solvent A (0.1% TFA) and solvent B (100% acetonitrile) at a flow rate of 1 ml per minute. The gradient was established such that B reached 17% in 10 minuted and was held at this value for 30 minutes. In the next 5 mins B reached 50% and in an additional 5 minutes 100%. HNA eluted at a retention time of 36 mins. The results showed apporx 40% decrease in HNA formation by the siRNA transfected lenses as compared to the untransfected lenses (Fig) due to which the unmetabolized HNE was higher in the siRNAtransfected lenses.

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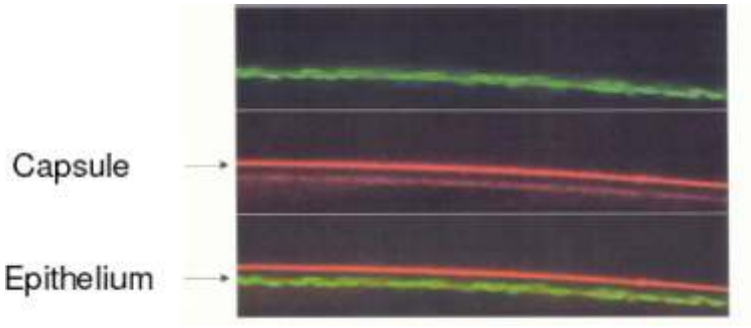

Figure 7: Transfection of rat lens with labeled Cy-3 dsRNA: Lens transfected with 100 pmoles of Cy3-dsRNA for 2 hrs. The transfecting media was changed to medium 199 and cultured overnight. These lens were counterstained with nuclear stain, Sito-16 for 30 mins before taking pictures on a Zeiss LSM-510 LSCM META using Achroplan 40 X 0.75 water immersion objective (LWD). Cy-3 dsRNA appears red and the nucleus green

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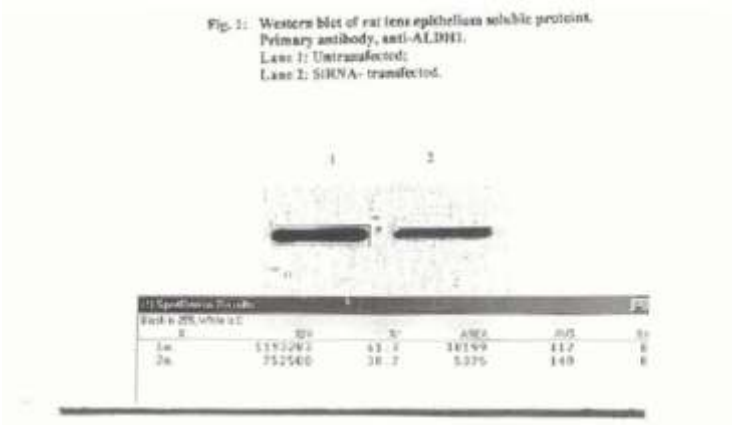
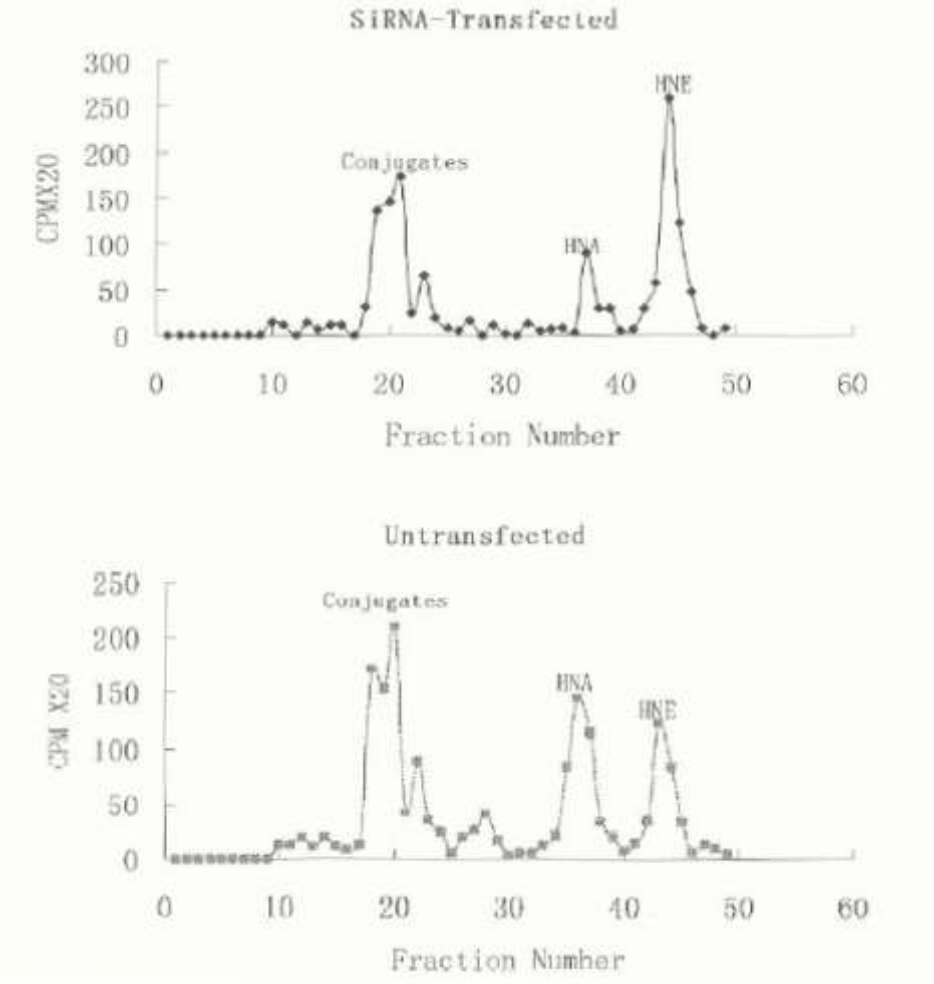


Fig. 2 Oxidation of HNE by untransfected and ALDH1-SiRNA-transfected rat lens



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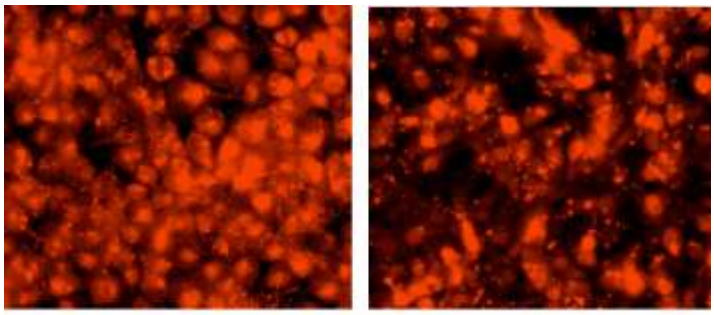

Figure 1: Transfection of Hela cells (left panel) and Cos-7 cells (right panel) with Cy3-dsRNA using the targefect siRNA transfection kit. This data has been kindly provided by Dr Yasuhito Abe, Ehime Univ. School of Medicine, Ehime, Japan

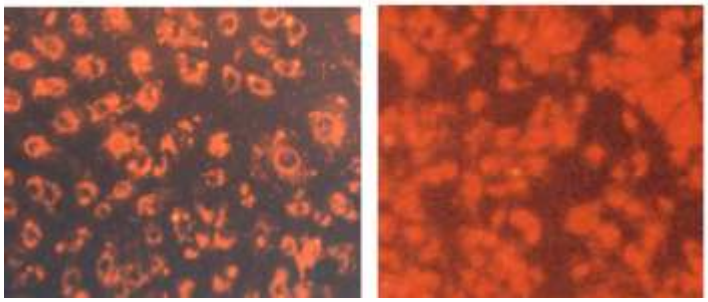

Figure 2: Left Panel- Transfection of OVCAR-3 cellswith Cy3-dsRNA (23 oligonucleotide doublestranded RNA); Right panel: Transfection of Cy3-DsRNA into HEK-293 cells

Advantages:

- Efficient gene silencing in many hard-to –transfect primary and established cell types.
- Sustained silencing of target gene expression by repeated siRNA transfections every 4 days.
- Applications for silencing genes both for in vitro and in vivo studies

Updated Transfection Protocol

General protocol:

Set up cells to be transfected so that they are about 70-90% confluent at the time of the experiment.

Prepare transfection complexes as follows:

Use clear plastic tubes for complex formation. If the siRNA reagents arrive frozen thaw them, mix well and store the siRNA reagents at 4 oC. **Please use high glucose DMEM (Dulbecco's modified eagel's medium containing 4500 mg/liter glucose) for preparing transfection complexes.**

Tube #	DMEM (high glucose)	dsRNA	Targefect soln A	Targefect Soln B
1	1 ml	75 pmols	5 ul	10 ul
2	1 ml	200 pmols	5 ul	10 ul
3	1 ml	75 pmols	5 ul	15 ul

Make aditions as follows: Add DMEM first, then add dsRNA, mix well by flicking the tube about 12 times to create a vortexing action. Add targefect soln A next, mix well again by flicking the tube and then add targefect soln B, mix well again. Incubate the tubes at 37 o C for 25 minutes to form the transfection complexes.

Wash cells to be transfected twice withDMEM. Aspirate the second wash completely. Add 1 ml of the transfection mix for 1 well for a 6-well dish (or for a 35 mm dish). Prepare 250 ul of transfection complex per well of a 12-well dish, 150 ul per well of

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a 24-well dish or 50 ul if using a 96-well plate Incubate the transfection complexes with the cells at 37 o C for 2 hrs. Add Complete media with serum (2 ml for a 35 mmm dish or one well of a 6-well plate, 1ml/well for a 12-well dish and 0.5 ml/well for a 24-well dish. Incubate overnight. Replace the media with fresh complete media the next morning and assy at 24-72 hrs post-transfection.

When using fluorescently labed dsRNA we have observed very efficient delivery into several cell types at 24 hrs post transfection and seen almost complete silencing of endogenous gene expression at 48-72 hrs post transfection as ascertained by western blot analysis.

For testing for silencing of transiently transfected genes we recommend transfecting the siRNA 12 hrs following transient transfection of the gene of interest and assaying for silencing of the transiently transfected gene 24 hrs after transfection of the siRNA.

For testing silencing of endogenous genes we recommend analysis 48-72 hrs after transfection of the siRNA.

Troubleshooting:

We have also observed that the regular protocl results in toxicity in certain cell types but this problem has been easily fixed by a few modifications in the few instances wehere this has been observed. Some labs have reported a littel initial toxicity but they say the cells recover very quickly and tehy get very good results on silencing.

Use of complexing media other than high glucose DMEM sometimes results in toxicity. The suggestions for reducing toxicity and obtaining complete silencing of endogenous gene exp[ression are as follows:

1) Try to work with almost confluent cells at the start of the experiment 90% confluent.

2) Decrease the incubation time with transfection complexes to 1.5 hrs instead of 2 hrs. If your complete media has very little serum (5% or less), we suggest aspirating of the transfection complex and then adding the complete media. Also adjusting the serum concentration to atleast 10% also helps

3) Perform experiment exactly as you did before using slightly more confluent cells and reducing the amount of solution B to 5 ul. Solution B is just an RNA stabilizing reagent. In some cell types the suggfested 15 ul soln B per ml of complex results in toxicity to the cells and reducing it to 5 ul per ml greatly decreases the toxicity. Also make sure you change the media the next morning after tranfection.

For Incomplete Silencing: Try two consecutive rounds of transfections with siRNA. (the second transfection should be performed within 24 hrs after the first transfection. Analyse for gene silencing at 72 hrs after the first transfection.