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Western Blot Evaluation of siRNA Delivery by pH Responsive Peptides

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Abstract

Gene silencing, via RNA interference (RNAi) technologies using small interfering RNA (siRNA), has been developed as an important tool for target identification and validation in drug discovery and has huge therapeutic potential. However, effective delivery into cells presents a major challenge to the use of siRNA. pH responsive cell-penetrating peptides have attracted considerable attention in recent years as delivery vectors due to their ability to transport their cargos across the biological membrane and/or to promote endosomal escape and prevent lysosomal degradation. To evaluate the *in vitro* transfection efficiency of the pH responsive peptide-based siRNA delivery system, the western blotting technique is commonly employed. This method offers a simple, efficient and economical way to study the gene silencing effect of the siRNA by analysing the protein of interest in a sample with minimum equipment requirement. This chapter provides a description of siRNA delivery and analysis by western blotting protocols for qualitatively and quantitatively assessing gene silencing efficiency and selectivity.

Keywords

Transfection; siRNA; GAPDH; Western blotting; Densitometry; pH responsive peptide; Chemiluminescence

1. Introduction

Substantial progress has been made in RNA interference (RNAi) technology in the last decade and small interfering RNA (siRNA) has become an important agent for target identification and validation in drug discovery (1). Tremendous effort has also been invested in recent years to develop siRNA-based therapeutics (2). Effective and non toxic delivery of siRNA into cells presents one of the major obstacles that hinder the use of siRNA in the drug discovery process and clinical applications (3). Being a negatively charged hydrophilic macromolecule that is highly susceptible to nuclease degradation, siRNA is incapable of crossing the biological membrane on its own to exert the gene silencing effect in the cytosol. A delivery vector is therefore required to protect the siRNA from enzymatic degradation, facilitate cellular uptake to the target cells and release the siRNA at the site of action. Although viruses are efficient nucleic acid delivery vectors in nature, safety concerns regarding their immunogenicity and toxicity have led to the development of safer non-viral vector alternatives. In recent years, cationic cell-penetrating peptides (CPPs) have attracted a

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lot of attention as siRNA delivery agents due to their ability to interact and cross cell membranes while binding to the negatively charged siRNA. CPPs are either covalently conjugated to siRNA through disulphide bond formation, or bind to siRNA through electrostatic interaction to form complexes in a non-covalent manner. The former approach may have a negative effect on cellular delivery as the biological activity of the peptides may be altered during the modification (4), whereas the latter approach offers a simpler way to achieve siRNA delivery and the peptide/siRNA complexes can be prepared easily. In addition, CPPs with pH responsive components can further enhance the intracellular delivery efficiency by promoting endosomal escape, thereby preventing the siRNA from degradation by enzymes in the late endosomes / lysosomes (5).

A good siRNA delivery system is crucial during the investigation of the gene silencing effect of siRNA whether it is used for target identification and validation or RNAi therapeutics. To evaluate siRNA transfection efficiency of a delivery system and the level of gene silencing *in vitro*, the western blotting technique is commonly employed as it offers a relatively simple, efficient and economical way to measure the expression of proteins of interest. Western blotting is a powerful tool to analyse protein samples from cells or tissues. Protein samples undergo electrophoresis and are transferred onto a nitrocellulose membrane. The transferred protein is probed with a specific primary antibody followed by an enzyme-conjugated secondary antibody. The activity of an enzyme such as horseradish peroxidase (HRP) is used for signal generation. The membrane is finally incubated with appropriate enzyme substrate to produce a recordable signal (Fig.1). Enhanced chemiluminescence (ECL) is one of the commonly employed methods that provide precise detection of proteins from western blots. In this method, HRP catalyses the oxidation of luminol into a reagent that emits light when it decays. Since HRP is complexed with the protein of interest on the membrane through antibodies, the location as well as the amount of light emitted is directly correlated to the location and amount of protein on the membrane. The light can be detected by photographic film or captured as digital image by CCD camera. The density of protein bands on the western blot image can be measured using appropriate gel image analysis software, and the gene silencing effect can be evaluated by comparing the relative protein expression of the sample with the untreated or negative control.

In this chapter, a pH responsive histidine-rich amphipathic peptide, LAH4-L1 (6-8), is employed as siRNA delivery vector to demonstrate the use of the western blotting technique to evaluate the *in vitro* siRNA-mediated gene silencing efficiency of a delivery system. We describe the entire procedure from the preparation of peptide/siRNA complexes, through the transfection of the cells, the techniques of western blotting, densitometry analysis of the blot to the calculation of gene silencing efficiency. siRNA targeting glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a housekeeping gene that is constitutively expressed at high levels in most cells and tissue, is used as positive control here. Non-targeting siRNA (a negative control) is also used to examine if the delivery agent has caused any effect to the cells. (Control RNAs are often selected on the basis that they produce limited off-target effects) (9).

2. Materials

2.1. Peptide and siRNA

1. pH responsive peptide: LAH4-L1 (available from e.g. EZBiolab Inc., USA; Pepceuticals Ltd, UK; ChinaPeptides Co., China). Re-suspend the peptide in filtered ultrapure water to give a stock solution with concentration of 2 mg/ml. Store in aliquots at -20°C .

2. siRNAs for western blotting experiment: Silencer® Select GAPDH siRNA for human, mouse and rat; Silencer® Select negative control siRNA for human, mouse and rat (Ambion). Re-suspend the siRNA in nuclease-free water to give a stock solution with concentration of 40 μ M. Store in aliquots at -20°C .

2.2. Cell culture

1. A549 cells (human lung adenocarcinoma). Subculture once a week, maintain in 5% CO_2 at 37°C .
2. Dulbecco's modified eagle medium (DMEM) supplemented with 10% FBS, 100 units/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin and 0.25 $\mu\text{g/ml}$ amphotericin B.
3. Transfection medium: Opti-MEM® I Reduced Serum Medium (Invitrogen).

2.3. Sample preparation

1. Cell lysis buffer stock solution (10 x): 1.21 g Tris (pH 7.4), 4.39 g NaCl, 0.19 g ethylenediaminetetraacetic acid (EDTA), 0.19 g ethylene glycol tetraacetic acid (EGTA), 0.28 g sodium pyrophosphate, 0.11 g β -glycerolphosphate, 0.09 g sodium orthovanadate and 5 ml Triton X-100. Make up to 50 ml with distilled water. Store at 4°C .
2. Cell lysis buffer (1 x): Dilute 100 μl of cell lysis buffer stock solution (10 x) to 1 ml with distilled water. Add 1 μl Leupeptin (5 mg/ml in distilled water), 1 μl Aprotinin (1 mg/ml in distilled water), 1 μl Pepstatin A (1 mg/ml in EtOH), 10 μl Dithiothreitol (15.4 mg/ml in distilled water) and 10 μl PMSF (17.4 mg/ml in isopropyl alcohol).
3. Sodium dodecyl sulphate (SDS) sample buffer stock solution: Dissolve 1.51 g Tris base to 15 ml of distilled water, and then add 25 ml of 50% glycerol into the mixture. Stir to dissolve and then adjust pH to 6.8 with HCl. Dissolve 5 g of SDS in this buffer and make up to 50 ml with distilled water. Dissolve 0.01 g of bromophenol blue and stir overnight.
4. Bradford protein assay reagent
5. Bovine serum albumin

2.4. SDS polyacrylamide gel electrophoresis (SDS-PAGE)

1. 30% Acrylamide:Bis Solution 37.5:1
2. Stacking gel buffer: 0.5 M Tris/HCl, 0.4% SDS. Weigh 6.06 g Tris base, 0.4 g SDS, make up to 100ml with distilled water and adjust pH to 6.8 with HCl. Filter and store at 4°C .
3. Resolving gel buffer: 1.5 M Tris/HCl, 0.4% SDS. Weigh 18.16 g Tris base, 0.4 g SDS, make up to 100ml with distilled water and adjust pH to 8.8 with HCl. Filter and store at 4°C .
4. Water saturated butanol
5. SDS-PAGE running buffer: Weigh 30.30 g Tris base, 144.0 g glycine, 10.0 g SDS, make up to 1000 ml with distilled water to give a 10 x running buffer stock solution. Dilute the running buffer stock solution using distilled water to 1 x before use.
6. 10% Ammonium persulfate (APS)
7. N, N, N, N'-tetramethyl-ethylenediamine (TEMED). Store at 4°C .

8. Pageruler™ prestained protein ladder
9. Electrophoretic blotting system
10. Electrophoresis power supply (EPS)

2.5. Electrophoretic transfer

1. Transfer membrane, nitrocellulose, 30 cm x 3 m roll, pore size 0.2 µm (Pall Corporation)
2. SDS-PAGE transfer buffer stock solution (10 x): Weigh 30.30 g Tris base, 144.0 g glycine. Make up to 1000 ml with distilled water.
3. SDS-PAGE transfer buffer (1 x): Dilute 100 ml of transfer buffer stock solution (10 x) to 800 ml with distilled water and then add 200 ml of methanol.
4. Tris buffered saline (TBS): Weigh 24.22 g Tris (0.2 M), 87.83 g NaCl (1.5 M), adjust pH to 7.4 and make up to 1000 ml with distilled water to give a 10 x TBS stock solution. Dilute the stock solution to 1 x with distilled water before use.
5. TBST: TBS containing 0.05% Tween-20
6. Blocking solution: 5% non-fat dry milk in TBS

2.6. Antibodies and conjugates

1. Primary antibodies: monoclonal anti-GAPDH antibody and monoclonal anti-β-actin antibody from mouse. (*see Note 1*)
2. Secondary antibodies: Amersham ECL™ anti-Mouse IgG, Horseradish peroxidase linked whole antibody.
3. Amersham ECL™ western blotting detection reagents

2.7. Densitometry analysis

1. Image J 1.45 software (NIH) (*see Note 2*)

3. Methods

3.1. Preparation of peptide/siRNA complexes for transfection

1. Dilute the stock solution of Silencer®Select GAPDH siRNA or Silencer®Select negative control siRNA to a concentration of 100 pmol/ 500 µl in Opti-MEM® I in a microcentrifuge tube.
2. Dilute the peptide stock solution in Opti-MEM® I Reduced Serum Medium to 500 µl in a microcentrifuge tube, so that the weight ratio of peptide to siRNA (prepared in step 1) is 10 to 1 (*see Note 3 & 4*).

¹Antibody dilution is established empirically. The dilution ratio is usually ranged from 1:500 to 1:10,000. The signal intensity should increase with increasing antibody concentration. Protein expression levels vary in different cell lines, therefore the dilution ratio of antibody should be optimized in accordance with each experiment/cell line to get satisfactory results. The final dilution also depends on the detection system used. Initially a lower dilution ratio (e.g. 1:500) can be tried. Also, monoclonal antibodies are recommended over polyclonal antibodies as the former are more specific and generally produce a cleaner background for protein analysis. Ensure the type of antibodies can target the species of the cells used for transfection.

²Image J is a public programme designed by National Institute of Health (NIH) USA for image processing and analysis. It can be downloaded for free from NIH website (<http://rsb.info.nih.gov/ij/>). There are many other software that can be used to analyse and quantitate protein expression on western blot including GelQuant, ImageQuant™ and GelEval™, some of which are supplied with gel documentation imaging system.

3. Add the peptide solution (500 μ l) to the siRNA solution (500 μ l) to give a final volume of 1 ml.
4. Mix the suspension by gently inverting the microcentrifuge tube several times. The mixture is incubated at room temperature for 30 minutes to allow the formation of stable peptide/siRNA complexes.

3.2. siRNA transfection and protein preparation for western blotting

1. Seed A549 cells in 6-well plates with a seeding density of 1×10^5 cells per well. (see Note 5)
2. After 24 hours, the cells are washed twice with warm PBS. Add the peptide/siRNA complexes prepared in Opti-MEM® I Reduced Serum Medium (as prepared in section 3.1) to the cells with a final volume of 1 ml per well (each well contains 100 pmol siRNA). (see Note 6)
3. The cells are incubated for 4 hours at 37°C, and are washed with warm PBS twice. Fresh DMEM supplemented with 10% FBS are added to the cells.
4. After 72 hours of incubation, the cells are washed with cold PBS twice. (see Note 7)
5. Scrape cells in 1 ml PBS (on ice). Transfer the cell lysate into a microcentrifuge tube which is centrifuged at 10,000 x g for 3 minutes at 4°C. Remove all the supernatant and add 100 μ l cell lysis buffer to the cells pellet.
6. Briefly sonicate the sample and centrifuge at 10,000 x g for 3 minutes at 4°C to remove the water-insoluble cell debris.
7. Protein concentration of the sample is quantitated by the Bradford protein assay with bovine serum albumin used as standard.
8. 20 μ g of protein from the cell lysate is transferred to a microcentrifuge tube and make up to 20 μ l with distilled water.
9. Add 5 μ l of 2-mercaptoethanol to 95 μ l of SDS sample buffer stock solution to give a sample buffer mixture. (SDS may precipitate at room temperature. Warm up the SDS sample buffer stock solution before use.) (see Note 8)
10. Add the protein sample to the sample buffer mixture at 4:1 volume ratio. Heat the samples at 95°C for 10 minutes. The samples are now ready for SDS-PAGE.

³The transfection efficiency and stability of some peptide/siRNA complexes may be affected by the presence of serum. It is suggested to prepare the complexes in the absence of serum initially. Serum-supplemented culture medium can be used with complexes that show good transfection efficiency in the absence of serum.

⁴The optimal weight ratio of LAH4-L1 to siRNA for transfection was determined previously as 10:1. A range of different ratios should be tried for other peptides if the optimal ratio has not been determined.

⁵Depending on the cell division rate and the adhesive property, different cell types may have different optimal cell seeding density for siRNA transfection. It is suggested to plate cells at densities ranging from 1×10^4 to 1×10^6 cells per well in a 6-well plate to find the optimal condition initially. If the cell density is too low, there may not be enough protein collected to perform western blotting analysis. Also, microplates with more wells than a 6-well plate are not recommended as there may not be enough protein collected for proper analysis.

⁶To optimize the amount of siRNA for *in vitro* transfection, prepare the peptide/siRNA complexes with various concentrations of siRNA, ranging from 1 pmol to 100 pmol per ml. The optimal amount of siRNA required for efficient gene silencing effects varies between the cell types used.

⁷Depending on the cell division rate, optimal gene silencing effects may be observed at different time points post-transfection. It is suggested to perform western blotting at 48 hour to 96 hour post-transfection to determine the optimal time point.

⁸2-mercaptoethanol is added to reduce the disulphide bond to disrupt the tertiary and quaternary structure of some proteins for analysis. It has a strong pungent smell. Handle sample containing 2-mercaptoethanol in a fume hood.

3.3. SDS polyacrylamide gel electrophoresis (SDS-PAGE)

1. Set up the gel apparatus. Clean the glass plates with 70 % ethanol and assemble them in a casting stand.
2. Prepare resolving gel mixture and stacking gel mixture as showed in table 1.
3. Pour the resolving gel mixture in between the glass plates and add sufficient amount of water saturated butanol on top to level off the gel. Wait for approximately 45 minutes.
4. After the resolving gel is ready, rinse with distilled water to remove butanol.
5. Add the stacking gel mixture on top and insert the gel comb. Wait for another 30 minutes. After the stacking gel is set, remove the comb and put the gel with the stand into the running tank.
6. Fill the running tank with cold SDS-PAGE running buffer. Carefully load the samples and the protein ladder into the wells. (*see Note 9*)
7. Run the samples at 120 volts, 500 mA for approximately 50 minutes. When the dye front reaches the end of the gel, switch off the power. Separate the gel plates by a spatula and remove the stacking gel.
8. Wash the gel (still supported by the bottom glass plate) carefully with distilled water.

3.4. Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose membrane

1. Cut a piece of nitrocellulose membrane to the size of gel.
2. Place nitrocellulose membrane, filter paper and fibrous pad in SDS-PAGE transfer buffer for at least 1 hour before use. All fibrous pad and filter papers should be totally wet before use.
3. Place the gel on a filter paper which has been soaked in transfer buffer.
4. Assemble the blotting sandwich cassette as follows (Fig.2): Support grid → fibrous pad → filter paper → gel → nitrocellulose membrane → filter paper → fibrous pad → support grid (*see Note 10*)
5. Close the clamp and place the cassette in gel blotting apparatus such that the membrane is close to the anode and the gel is close to the cathode.
6. Fill the tank 3/4 full with SDS-PAGE transfer buffer, put a stir bar into it and install the frozen ice pad. Top up with buffer, turn on magnetic stirrer and close the lid.
7. Run the blot at maximum voltage with a constant current of 300 mA for 90 minutes. High molecular weight protein may need longer transfer time.
8. After transfer is complete, shut off power and remove cassette holder.
9. Mark the membrane with a ballpoint pen for sample identification purpose. Do not let the membrane dry.

⁹Different proteins have dissimilar expression in differing cell types. To get a good signal for analysis, the total amount of protein loaded into each well should be optimized for different cell type.

¹⁰It is important to avoid air bubbles when assembling the blotting sandwich cassette in order to allow proteins to be transferred properly to the nitrocellulose membrane.

10. Minimize the background signal by blocking the membrane in 5% non-fat dry milk blocking solution for 2 hours with shaking at room temperature. (see Note 11)
11. Remove the blocking solution and wash the membrane three times with TBST.

3.5. Antibodies probing (anti-GAPDH)

1. The membrane is incubated with primary antibody (anti-GAPDH) overnight at 4°C on shaking.
2. Quickly rinse the membrane with TBST followed by washing three times with TBST for 10 minutes each.
3. Incubate the membrane with horseradish peroxidase conjugated secondary antibody for 2 hours at room temperature on shaking.
4. Rinse the membrane two to three times followed by 10 minutes wash twice with TBST and 10 minutes wash twice with TBS (large volume). Remove buffer.

3.6. ECL detection

1. Add ECL reagent mixture on the membrane for exactly 1 minute.
2. Blot the membrane dry with soft tissue papers. Wrap the membrane in plastic wrap.
3. Expose the wrapped membrane to the film in ECL cassette and develop the film in dark room: 1 minute in developer, 1 minute in water, 1 minute in fixer and 1 minute in water. Repeat the ECL exposure if necessary. (see Note 12 & 13)
4. After developing the film, overlay the film and nitrocellulose membrane to confirm the molecular weight of the band with the aid of a protein ladder (Fig 3). Identify the target band according to the molecular weight of the protein.
5. If a gel documentation imaging system with CCD camera installed is available (which can be expensive), the light output of the membrane can be captured automatically without the need of developing the film in dark room. (see Note 14)

3.7. Probing with another primary antibody (anti-β-actin)

1. To detect another protein expression, the membrane is rinsed twice with TBST and incubated with another primary antibody (anti-β-actin) for 1 hour at room temperature on shaking.
2. Quickly rinse the membrane with TBST followed by 10 minutes wash three times with TBST.

¹¹The purpose of blocking the membrane is to minimize any non-specific binding which may give rise to background, affecting the protein analysis at later stage. Apart from 5% non-fat dry milk in TBS, 3-5% bovine serum albumin in TBS is an alternative blocking solution that is commonly used.

¹²Since different proteins have distinct expression levels in each cell line, the exposure time of protein should be adjusted accordingly. If the expression of a particular protein in a particular cell line is very weak, the exposure time of this protein can be increased to improve the intensity of signal. However the background signal will increase with extending of exposure time. If exposure time has been prolonged and the signal is still too weak to be detected, then increasing total amount of protein loaded into the well should be considered.

¹³Beside normal ECL detection reagent, ECL prime (Amersham) is one of the latest ECL products for chemiluminescent detection of western blotting and provides higher sensitivity over the normal ECL reagent. If the concentration of target protein is too low and the primary antibody is limited, this product may be considered as an alternative.

¹⁴If a gel documentation imaging system with CCD camera installed is available, chemiluminescence can be captured by such a system after the nitrocellulose membrane is exposed to ECL. Definite time intervals can be set and the camera is able to record the signal continuously to accumulate the signal automatically. The image which has optimal signal intensity can be selected and there is no need to adjust the exposure time. This is the key advantage of such an imaging system.

3. Incubate the membrane with horseradish peroxidase conjugated secondary antibody for 1 hour at room temperature on shaking.
4. Rinse the membrane twice followed by 10 minutes twice with TBST and another 10 minutes wash twice with TBS (large volume). Remove buffer.
5. Repeat section 3.6 to detect the expression of β -actin.
6. Since the molecular weight of GAPDH is around 36 kDa and β -actin is about 42 kDa, their bands appear in distinct areas of the membrane and are unlikely to interfere with each other. However if the two or more proteins to be detected on the same membrane have a similar molecular weight, stripping and re-probing is necessary. (see Note 15)

3.8. Western blot image

1. From the western blot image (Fig. 4), the GAPDH protein band of the untreated sample and the negative control sample (in which the Silencer® Select negative control siRNA is used) should give the highest intensity, as the GAPDH protein is not silenced. For LAH4-L1/siRNA complexes treated samples (Silencer® Select GAPDH siRNA is used), the intensity of the GAPDH protein band should decrease as the amount of siRNA per well increases, indicating the increase of gene silencing efficiency.
2. β -actin protein (not targeted by siRNA) is used as the internal control to ensure that the amount of total protein added per well is the same, therefore the band intensity of β -actin should be about the same among all samples. To calculate the gene silencing efficiency (as shown in section 3.9), the GAPDH expression for each sample is normalised with the β -actin expression of the corresponding sample.

3.9. Densitometry and protein band analysis

1. Open the Image J software and the western blot image (scanned from the film or captured by a camera). (see Note 16 & 17)
2. Draw a rectangle around each single band. The size of rectangle should be large enough to encompass the entire band. The dimension of the rectangle should be kept the same for all other bands to be analysed in the same experiment. (Fig.5)
3. Create a profile plot which represents the relative density of the contents of the rectangle over each band (Fig 6). The peak corresponds to the dark bands in the rectangle. The area under the curve (AUC) is proportional to the intensity of the band.
4. To calculate the gene silencing efficiency of the peptide/siRNA complexes, the expression of the protein of interest (in this case: GAPDH) has to be first normalised with the expression of β -actin (protein for internal control) of the same sample.

¹⁵Stripping and re-probing membranes: wash membrane with TBS. Soak membrane in pH2 TBST for 5 minutes. Rinse the membrane and soak it in pH2 TBST for further 5 minutes. Wash membrane with TBST twice. Block membrane and repeat antibodies probing (section 3.5)

¹⁶Image J supports various formats of image, including TIFF, GIF, JPEG PNG, BMP and FITS. Since JPEG files use compression that will lead to loss of original data, it is recommended to use JPEG files only for illustration purpose since they may not be suitable for quantitative analysis. This method described here is modified from the gel analysis procedure from Image J manual. There are many ways to perform densitometric analysis. Similar results should be achieved with different methods.

¹⁷Images of the blots sometimes have background noise. This can be compensated for by subtracting the background signal from the signal of the band.

The normalised intensity of GAPDH of sample treated with siRNA targeting GAPDH, is compared with the normalised intensity of GAPDH of sample treated with negative control siRNA (equation 1). The percentage is the remaining expression of GAPDH of the sample after transfection with peptide/siRNA complexes. The data is represented in a bar chart (Fig.7). Experiments are repeated independently at least three times for detection of significant differences in silencing activity.

$$\frac{\text{Intensity of GAPDH}_{(\text{sample treated with GAPDH siRNA})} / \text{Intensity of } \beta\text{-actin}_{(\text{sample treated with GAPDH siRNA})}}{\text{Intensity of GAPDH}_{(\text{sample treated with negative control siRNA})} / \text{Intensity of } \beta\text{-actin}_{(\text{sample treated with negative control siRNA})}} \times 100\% \quad \text{Equation 1}$$

Equation 1. Formula to calculate gene silencing efficiency of the delivery system. The percentage represents the remaining GAPDH protein expression after transfection.

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Figure 1. Schematic diagram to illustrate the principle of western blotting in generating signals from target proteins through antibody probing.

Figure 2. Illustration of the setup of the sandwich cassette in the transfer tank observed from the top for the western blotting experiment.

Figure 3. Identification of the target protein (GAPDH) by comparing the molecular weight of the band on the film with the protein ladder with known molecular weight on the nitrocellulose membrane.

Figure 4. Western blot image of GAPDH expression in A549 cells at 72h post-transfection with LAH4-L1/siRNA complexes. The intensity of GAPDH protein band decreases as the amount of siRNA per well increases. For the negative control (– ve), 100 pmol siRNA/well was used. β -actin was used as internal control.

Figure 5.
Rectangles are drawn around all the individual bands to be analysed. All the rectangles encompass the entire band patterns and all rectangles are of the same dimension.

Figure 6.
The profile plots of the eight samples. The peak of each plot represents the relative density of the band in each rectangle. The larger the AUC, the higher the intensity of the band.

Figure 7.

Gene silencing efficiency in A549 cells analysed by western blotting. Cells were transfected with LAH4-L1/siRNA complexes containing GAPDH siRNA of increasing amounts (from 1 to 100 pmol per well). Protein analysis was carried out at 72 h post-transfection. The level of GAPDH protein expression of each sample was compared with the cells transfected with negative control siRNA.

Table 1

The components of the resolving gel and stacking gel for separation of proteins with different molecular weights.

	Resolving gel			Stacking Gel
	7.5 % Proteins >100 kDa	10 % Proteins 30-100 kDa	12.5 % Proteins 10-20 kDa	
30% Acrylamide/bis (29:1)	2.5 ml	3.4 ml	4.0 ml	1.3 ml
Resolving/Stacking gel buffer	2.5 ml	2.5 ml	2.5 ml	2.5 ml
Distilled H ₂ O	5.0 ml	4.1 ml	3.5 ml	6.0 ml
10 % APS (fresh)	50 µl	50 µl	50 µl	30 µl
TEMED	5 µl	5 µl	5 µl	10 µl