3. siRNA PROFILE AND ANALYTICAL METHOD DEVELOPMENT

siRNA targeting Fibroblast Growth Factor-2 (FGF-2) was selected to knockdown FGF-2 gene expression which is up-regulated in pulmonary arterial hypertension. FGF-2 siRNA for targeting FGF-2 gene of different species i.e. bovine and rat were used, as *in vitro* cell line studies will be carried out with the use of bovine FGF-2 siRNA and rat specific FGF-2 siRNA will be utilized to carry out *in vivo* studies in rats (1-3). siRNA is available as predesigned siRNA molecules as well as custom synthesized 21 mer or 27 mer duplexes. Two purification grades are generally available which includes desalted and HPLC grade. Our siRNA was procured as a ready to use HPLC grade duplex with 1 ml 5x siMAX^{TM} buffer (30mM HEPES, 100 mM KCl, 1mM $MgCl_2$, pH = 7.3).

Functionality of siRNA is based on some basic requirement which majorly includes biophysical or thermodynamic properties and Guanine/cytosine (G/C) content of each duplex. Most highly functional siRNAs (having functionality more than 95%; ≥F95) had a G/C content in range of between 36% and 52%. The G/C content groups bracketing the 36–52% contained an increased proportion of nonfunctional siRNAs; thus, a 36–52% G/C content was selected as criterion I for siRNA functionality, consistent with previous findings (4). Application of this criterion alone provided only a marginal advantage for selecting functional siRNAs from the panel. The relative stability and propensity to form internal hairpins can be estimated by the predicted melting temperatures (T_m) (5, 6). Sequences with high T_m values would favor internal hairpin structures. Sorting the functional siRNA classes by T_m shows that duplexes lacking stable internal repeats were better silencers (no F95 duplexes exhibited $T_m > 60°C$ or predicted hairpin structures). By considering these basic criteria, we have selected the siRNA for our research work.

Furthermore, the obtained siRNA was also analysed by Matrix Assisted Laser Desorption Ionization- Time of Flight-Mass Spectrometry (MALDI-TOF) and capillary gel electrophoresis to find out the molecular weight and purity of the received siRNA, respectively. Selected siRNA was ionized by for mass spectrometry using MALDI. It is a soft ionization technique used in mass spectrometry, allowing the analysis of biomolecules (biopolymers such as proteins, peptides and nucleic acids) and large organic molecules which tend to be fragile and fragment heavily when ionized by more conventional ionization methods like electron spray ionization. MALDI is a two-step process involving firstly the UV laser beam triggered desorption and ionization. Matrix material heavily absorbs UV laser light, leading to the ablation of the biomolecules with matrix material form upper layer

(~1 μm) in a hot plume. The hot plume produced may contain neutral and ionized matrix molecules, protonated and deprotonated matrix molecules, matrix clusters and nano-droplets. These matrix molecules in turn ionize biomolecules to be analysed, mainly through protonation or deprotonation (7). These ionized biomolecules are further analysed for mass-to charge (m/z) ratio. MALDI analysis was done by MALDI-TOF-MS.

Capillary Gel Electrophoresis (CGE) is a variant of conventional gel electrophoresis which is used to determine the purity of siRNA. It uses capillary filled with polymer solution to obtain a molecular sieve. The mechanism involves separation of analytes having similar m/Z ratios based on size. This method is commonly employed in Sodium Dodecyl Sulfate (SDS)-Gel molecular weight analysis of proteins and has also found application in DNA sequencing and genotyping (8). Capillary electrophoresis also provides an effective alternative to manual slab gel electrophoresis due to automation, quantitation, high speed and efficiency. Biomolecules like proteins, carbohydrates and nucleic acids can be separated using capillary gel electrophoresis. SDS-bound proteins with constant m/z are separated according to difference in protein molecular size. Multiple Capillary Gel Electrophoresis (mCGE) was carried out by using a cePRO 9600 machine.

3.1 Bovine FGF-2 siRNA

Guide strand sequences (5' → 3'): CCGUUACCUUGCUAUGAAA

- ✓ MW [g/mol]: 13285
- ✓ T_m [°C]: 54.0
- ✓ Purification: HPLC
- ✓ GC-Content [%]: 38.1

Sense-strand Analysis

- ✓ Sequence: 5'-CCG UUA CCU UGC UAU GAA A-3'
- ✓ Sense: 5'- [CCGUUACCUUGCUAUGAAA] RNA [TT] DNA -3'
- ✓ Target mass of the sense strand: 6591 Da
- ✓ Detected mass of the sense strand: 6589 Da

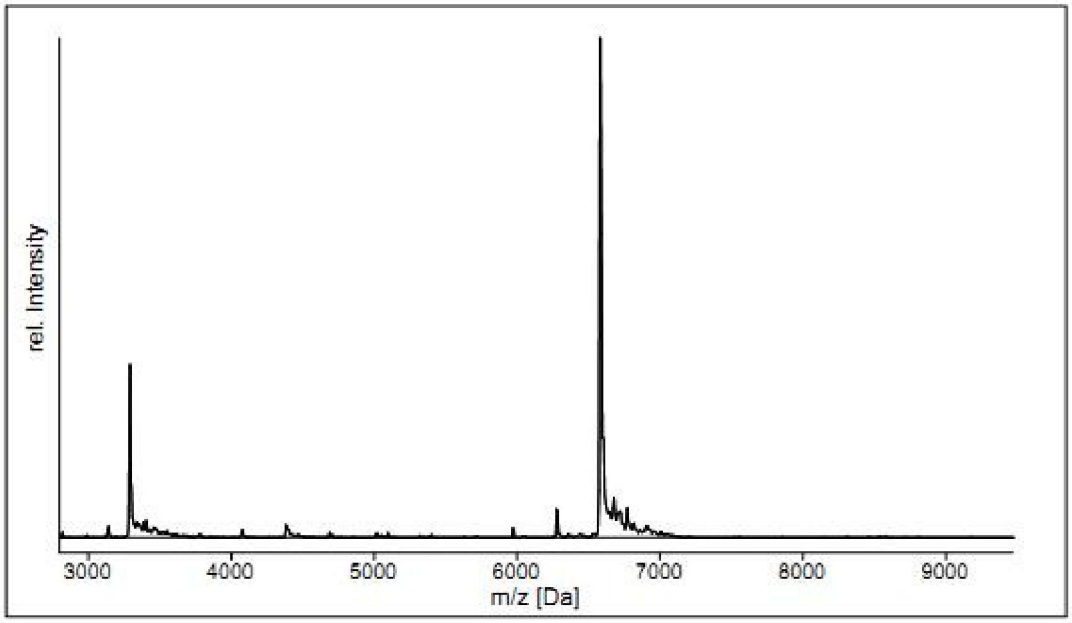


Figure 3.1 MALDI-Mass spectrometry of sense strand of Bovine siRNA.

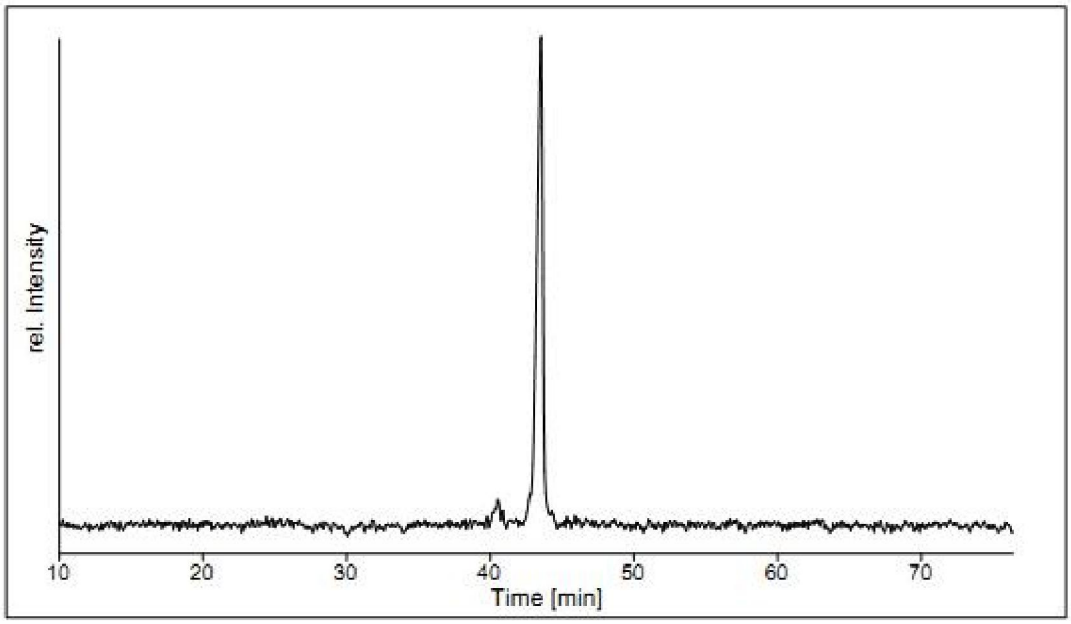


Figure 3.2 Capillary Gel Electrophoresis (CGE) analysis of sense strand of Bovine siRNA.

Antisense Strand Analysis

- ✓ Sequence: 5'-CCG UUA CCU UGC UAU GAA A-3'
- ✓ Antisense: 5'- [UUUCAUAGCAAGGUAACGG] RNA [TT] DNA -3'
- ✓ Target mass of the antisense strand: 6694 Da
- ✓ Detected mass of the antisense strand: 6692 Da

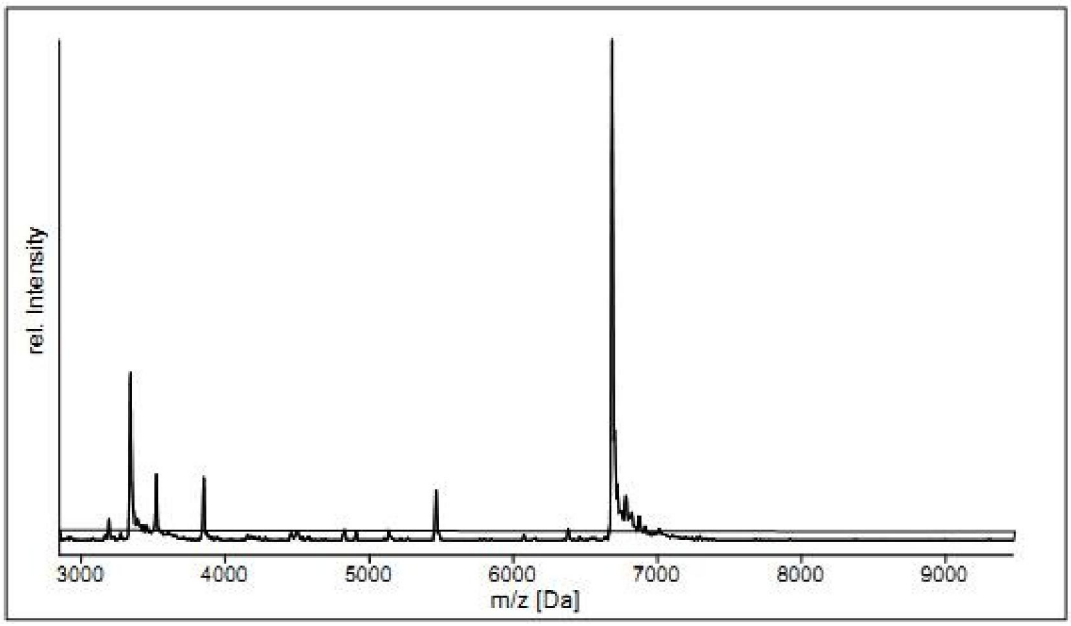


Figure 3.3 MALDI-Mass spectrometry of antisense strand of Bovine siRNA.

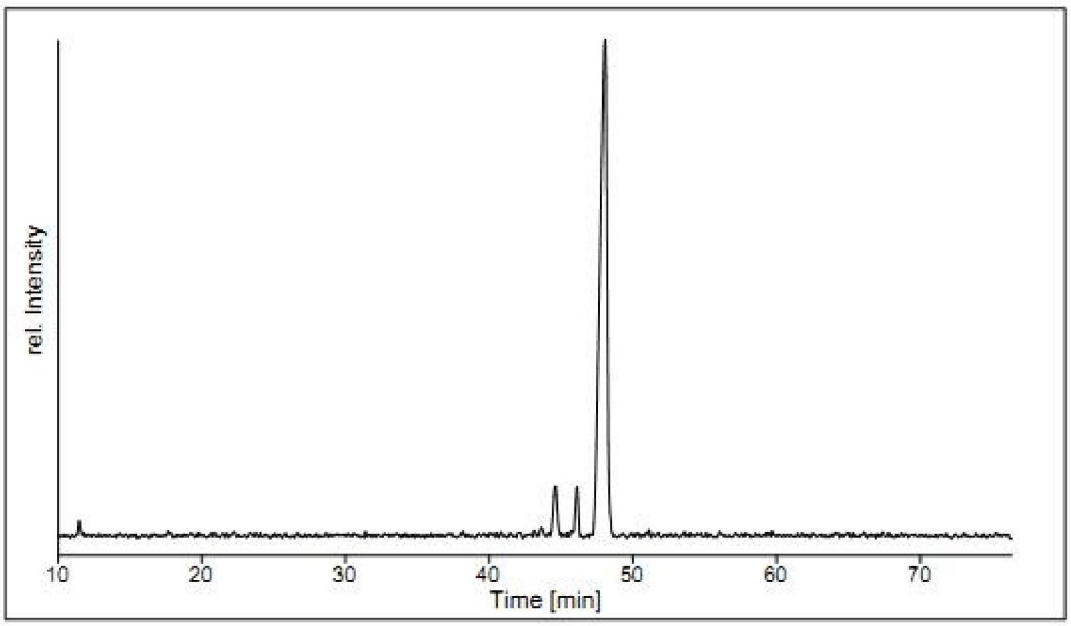


Figure 3.4 Capillary Gel Electrophoresis (CGE) analysis of antisense strand of Bovine siRNA.

3.2 Rat FGF-2 siRNA

Guide strand sequences (5' → 3'): AUA CUC CAG UUG GUA UGU G

- ✓ MW [g/mol]: 13285
- ✓ T_m [°C]: 54.0
- ✓ Purification: HPLC
- ✓ GC-Content [%]: 38.1

Sense Strand Analysis

- ✓ Sequence: 5'-AUA CUC CAG UUG GUA UGU G-3'
- ✓ Antisense: 5'- [AUACUCCAGUUGGUAUGUG] RNA [TT] DNA -3'
- ✓ Target mass of the antisense strand: 6648 Da
- ✓ Detected mass of the antisense strand: 6648 Da

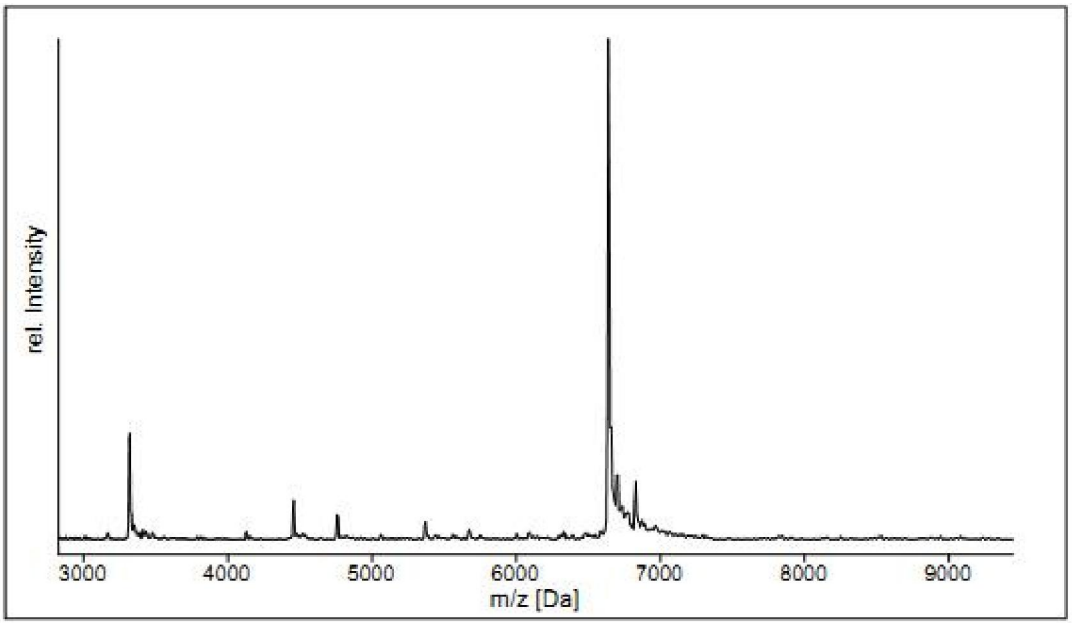


Figure 3.5 MALDI-Mass spectrometry of sense strand of Rat siRNA.

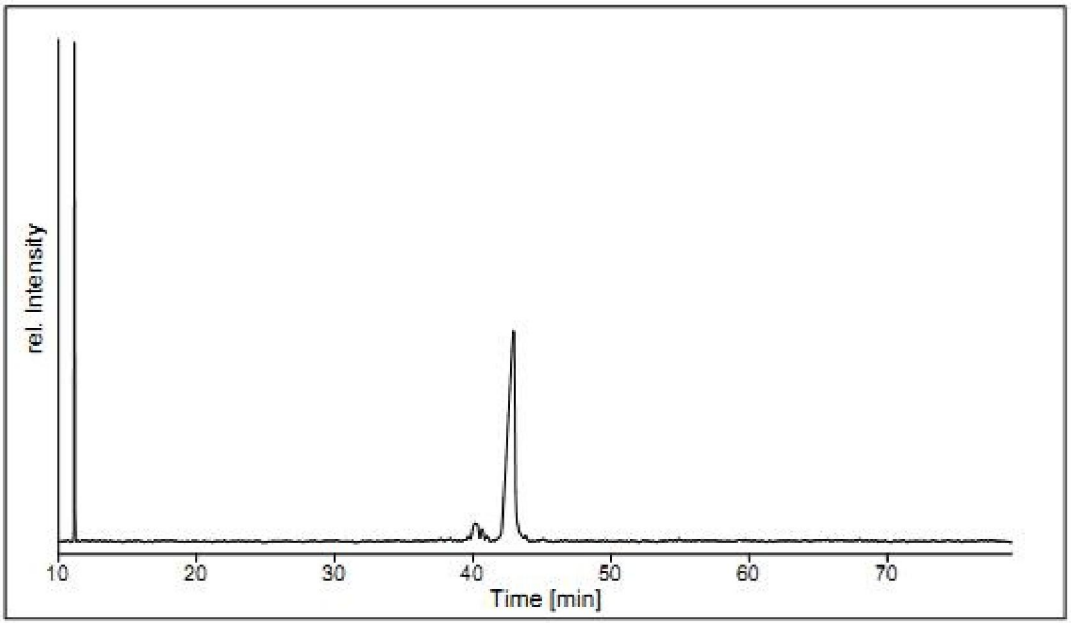


Figure 3.6 Capillary Gel Electrophoresis (CGE) analysis of sense strand of Rat siRNA.

Antisense Strand Analysis

- ✓ Sequence: 5'-AUA CUC CAG UUG GUA UGU G-3'
- ✓ Antisense: 5'- [CACAUACCAACUGGAGUAU] RNA [TT] DNA -3'
- ✓ Target Mass of the antisense strand: 6637 Da
- ✓ Detected Mass of the antisense strand: 6637 Da

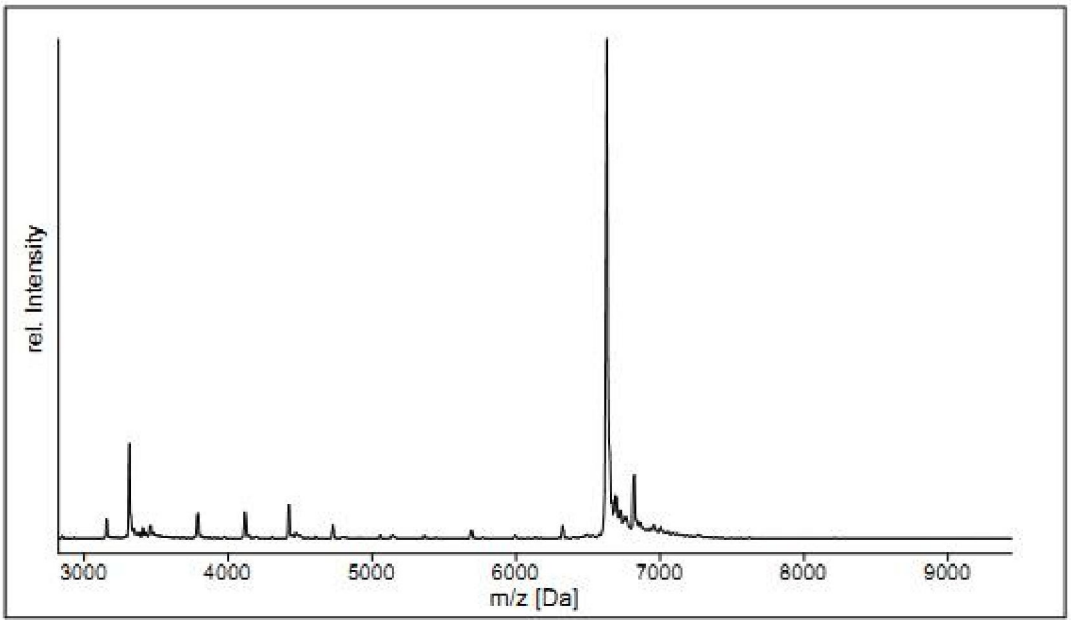


Figure 3.7 MALDI-Mass spectrometry of antisense strand of Rat siRNA.

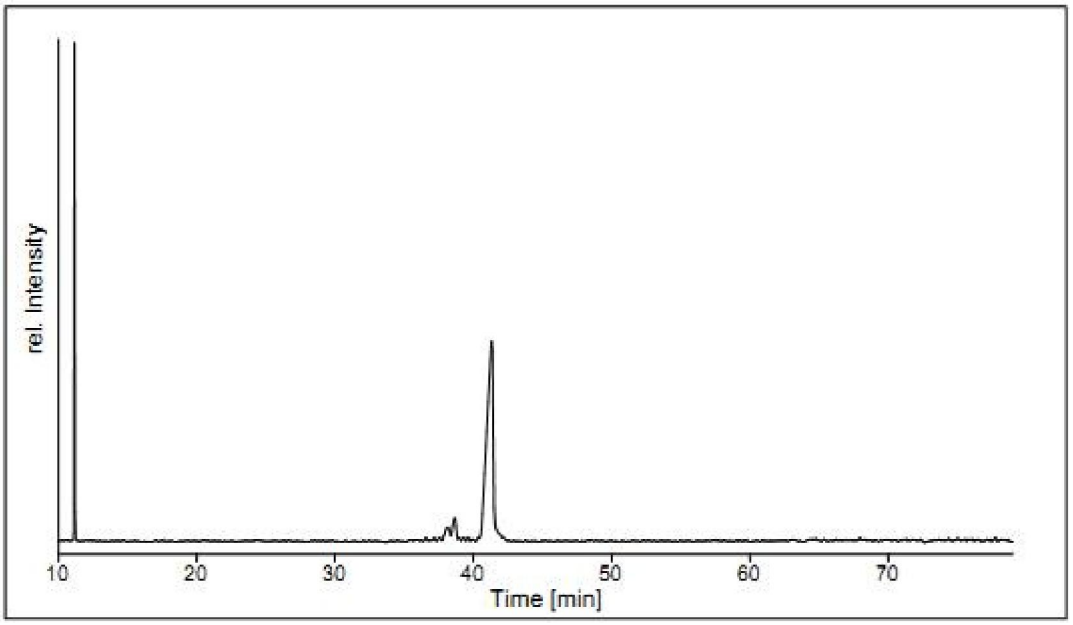


Figure 3.8 Capillary Gel Electrophoresis (CGE) analysis of antisense strand of Rat siRNA.

Results of MALDI-TOF-MS and CGE of sense and antisense strand of both the siRNA (**Figure 3.1, Figure 3.2, Figure 3.3, Figure 3.4, Figure 3.5, Figure 3.6, Figure 3.7** and **Figure 3.8**) confirmed purity and molecular weight of both FGF-2 siRNA.

3.3 UV Spectrophotometric Analysis of siRNA

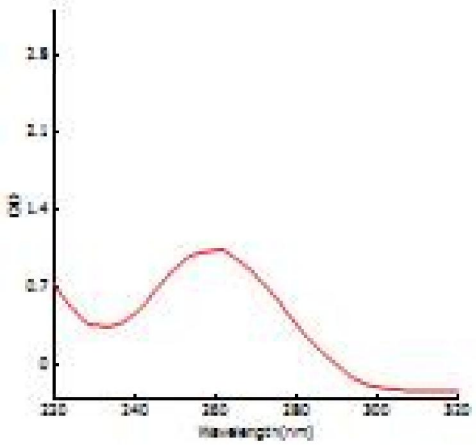
siRNA was quantified using a UV spectrophotometric method (9, 10).

Preparation of Nuclease Free Water: 1 ml of Diethyl Polycarbonate (DEPC) was mixed with 1 L doubled distilled water and stirred overnight on magnetic stirrer. Prepared dispersion was then autoclaved at 121°C and 15 Psi for 15 min to prepare nuclease free water.

Apparatus were washed by DEPC water to neutralize DNAses and RNAses. Purity of the siRNA was checked by taking the ratio of A260/A280 and A260/A230. Once siRNA was confirmed for its purity then calibration curve was constructed using this siRNA. siRNA stock solution of 100 pmole/µL was prepared in nuclease free water (NFW) and by appropriate dilutions of stock solution with NFW, siRNA solutions of various concentrations were prepared. Absorbances of these solutions as well as stock solution were recorded at 260 nm on a NanoDrop UV spectrophotometer. Whole experiment was performed in triplicate. Graph of observed concentration Vs actual concentration of siRNA was plotted to find out linearity of specific concentration range and reproducibility of results.

Ultraviolet absorbance can be used to measure DNA, RNA or protein concentration. For siRNA, the three main wavelengths of interest were 260nm, 280nm and 230nm. Absorbance at 260 nm was used to measure the amount of siRNA present in the sample. Concentration was calculated using the reading at 260nm wavelength and a conversion factor based on the extinction coefficient for nucleic acid. Simultaneously, absorbance measurements at that wavelength on 280 nm was used to estimate the amount of protein contaminant in the sample based on presence of aromatic amino acids which absorb light at 280 nm. Measurement at 230 nm was used to determine the amount of other contaminants that may be present in the samples. In addition, an absorbance reading at 320 nm was taken to detect any light-scattering components in the sample. The reading at 320 nm wavelength was subtracted from the 260nm, 280nm and 230nm values as background automatically by the software.

To estimate purity of siRNA, the ratio of the A260 with A280 and A230 were considered as key parameters. However, typical requirements for A260/A280 ratios are 1.8–2.2, while requirements for A260/A230 ratios are generally >1.7 (11). A260/A280 and A260/A230 values were 2.14 and 2.22 respectively as shown in **Figure 3.9** suggested good purity of siRNA. This could also be due to the use of synthetic siRNA of good purity. This siRNA was then used to verify the correlation of actual concentrations and observed concentration by NanoDrop (**Figure 3.10**). Manufacturer of NanoDrop UV Spectrophotometer provided the specified sample range of 2-100 ng/µL for reproducible results. This was further used to record the range of linearity which will be useful in determination of siRNA concentration in further studies.



Item	Result
OD260	1.030
OD280	0.358
OD230	0.351
OD320	-0.238
Pathlength (mm)	0.719
Dilution	1.000

Figure 3.9 Results generated from NanoDrop.

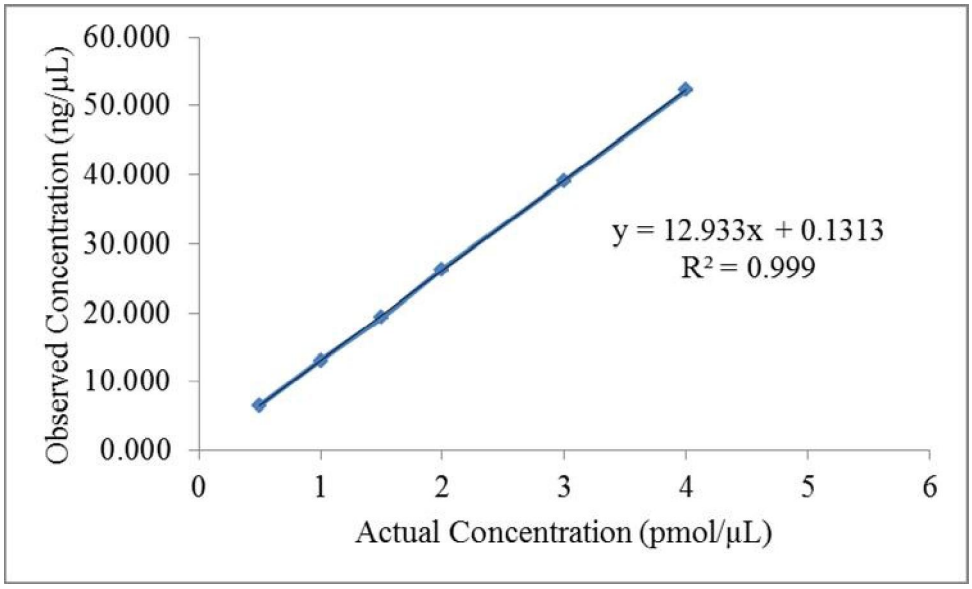


Figure 3.10 Correlation of actual concentration of siRNA Vs observed concentration by NanoDrop.

Results of known dilution of siRNA solution gave the good linearity and reproducibility. This range of linearity will be used in the further studies to find out the concentration of siRNA.

3.3.1 Accuracy and Precision

UV-spectrophotometric method using NanoDrop was evaluated for precision and accuracy in terms of % recovery and relative standard deviation (%RSD) respectively. siRNA standard solutions of 0.5, 2 and 4 pmole/µL were prepared covering the concentration range expected in samples, using nuclease-free water. Absorbance of each solution was recorded and % recovery was calculated. The reproducibility (Precision) of the method was determined by measuring absorbance of each sample at different time periods and calculating % relative standard deviation. All the measurements were made in triplicate in **Table 3.1** and **Table 3.2** represent accuracy, intraday and interday precision of the method respectively. As it can be seen, the % recovery was found to be between 98.0% to 100.0% and the % RSD values were less than 2% as per the requirements of ICH guidelines (12, 13).

Table 3.1 Results of accuracy measurements

Actual Concentration (pmole/µL)	Observed Concentration (ng/µL)	Standard Deviation (SD)	%Recovery
0.5	6.55	0.075	98.61
2	26.21	0.114	98.64
4	52.30	0.265	98.41

*Values are represented as mean±SD, n=3.

Table 3.2 Interday and intraday precision of the method

	Observed Concentration±SD		%Relative Standard Deviation	
Actual Concentration (pmole/µL)	Intraday precision	Interday Precision	Intraday precision	Interday Precision
0.5	6.55±0.075	6.48±0.071	1.158	1.032
2	26.21±0.114	26.17±0.133	0.436	0.748
4	52.30±0.265	52.19±0.175	0.508	0.259

*Values are represented as mean±SD, n=9.

3.4 siRNA Gel Electrophoresis: Gel Retardation Assay

Agarose gel electrophoresis was used for relative quantification of free siRNA migrated on the gel due to their charge under influence of electric potential (14). Cationic delivery vectors for siRNA should be able to complex with negatively charged siRNA. Polyethylenimine modified with Boc protected amino acids (Boc-amino acids) and cationic liposomes were prepared to complex with siRNA effectively to make them useful for successful delivery of siRNA. To test the complexation capacity of polymers and cationic lipids, agarose gel electrophoresis assay was performed by using ethidium bromide (EtBr) as a RNA binding dye. This is a molecular biology technique that is used to separate nucleic acids by size. Nucleic acids, DNA and RNA, migrate through an agarose gel matrix to positive electrode depending on the size of the molecule. The smaller the molecules are, the farther they will migrate on the gel. To visualize nucleic acids on gel, fluorescent dye like ethidium bromide is commonly used which intercalates into the minor groves of double-stranded nucleic acid strands.

As described earlier, free siRNA will migrate on the agarose gel, but if siRNA has been complexed with cationic polymer or phospholipids, such migration would be retarded. And the quantity of the migrated free siRNA would give a direct idea of the quantity of complexed siRNA.

3.4.1 Method of Analysis

Agarose (2 g, electrophoresis grade) was dissolved in 100 ml of 1x TBE (Tris-Borate-EDTA) buffer by heating on a heating mantle with intermittent shaking until the clear solution obtained. Melted agarose was allowed to cool to a pourable consistency. Till then, the gel tray was tightly secured at both the ends with tape to fluid-tight seal and comb was placed in the gel casting tray. Ethidium bromide was added (0.5 µg/mL) to the cooled agarose (to about 60°C) and mixed properly. Ethidium bromide loaded gel was poured in the gel casting tray up to 5 mm height. Gel was allowed to set at 20°C for 30 min followed by 15 min refrigeration for complete solidification of gel. Comb was removed from solidified gel and tapes were taken off the edges of gel tray. Gel was submerged in electrophoresis chamber (Genet Electrophoresis Powerpack, Banglore, India) with electrophoresis buffer (1x TBE buffer) (**Figure 3.11**).

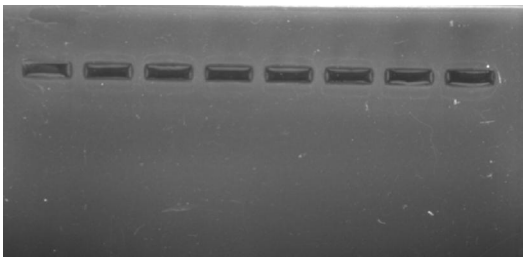


Figure 3.11 Agarose gel.

Initially, limit of quantitation of the gel electrophoresis method for siRNA detection was evaluated using 2% agarose gel in order to estimate minimum quantity that can be easily determined and hence further calibration range can be determined and evaluation can be performed in minimum quantity of siRNA formulation (**Figure 3.12**).

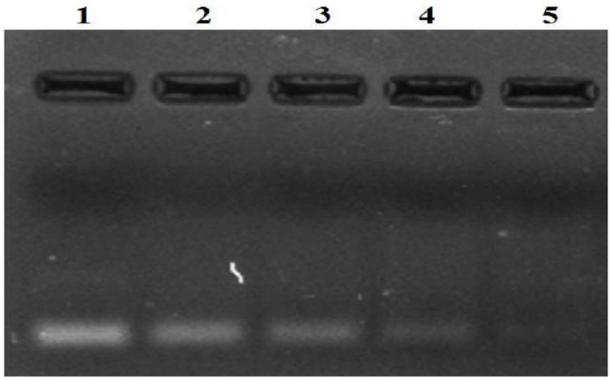


Figure 3.12 Estimation of minimum quantity of siRNA by gel retardation assay.
Lane 1: 50 pmol; Lane 2: 40 pmol; Lane 3:30 pmol; Lane 4: 20 pmol; Lane 5:10 pmol.

3.4.2 Determination of Quantifiable Range of siRNA for Gel Retardation Assay

For determination of quantification range for siRNA, siRNA solutions of different concentration (10, 20, 30, 40, 50, 75, 100, and 200 pmole) were prepared and evaluated by gel electrophoresis. For loading, siRNA solutions were mixed priorly with gel loading buffer (sucrose 50% w/v + bromophenol blue 0.25% w/v) in 0.5 mL micro-centrifuge tubes. 15 µL of each siRNA solution was loaded in to the wells and electrophoresed at 100 V/cm voltage (Genet Electrophoresis Powerpack, Banglore, India) for 20 mins. Post-run, gel was removed and migrated siRNA was visualized under UV light using GelDoc™ XR^+ Imaging System (BioRad, USA).

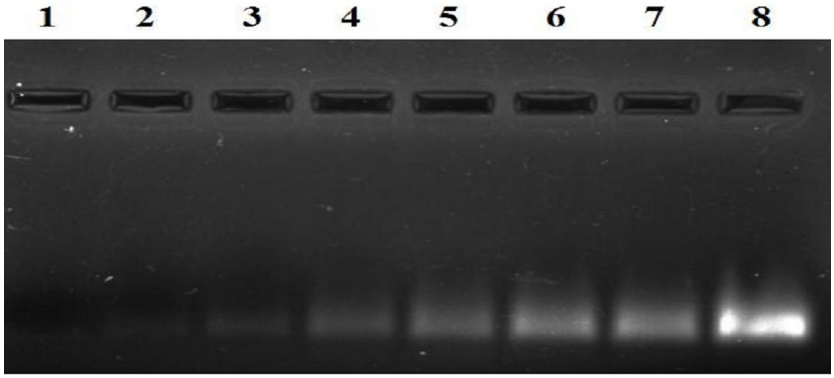


Figure 3.13 Determination of quantifiable range of siRNA - gel electrophoresis band densities at different siRNA concentrations.
Lane 1- 10 pmole, Lane 2- 20 pmole, Lane 3- 30 pmole, Lane 4- 40 pmole,
Lane 5- 50 pmole, Lane 6- 75 pmole , Lane 7- 100 pmole, Lane 8- 200 pmole.

UV-visualized gel (**Figure 3.13**) show that ≥ 30 pmole concentrations of siRNA are detectable and quantifiable, while lower concentrations band intensity was not appropriately quantifiable.

3.4.3 Relative Quantification

Calibration curve for relative quantification of siRNA was constructed by taking the band density at 50 pmole concentration as 1 and evaluating other band densities relative to that of 50 pmole concentration. Briefly, siRNA solutions of different concentrations (30-50 pmoles) were prepared and mixed with gel loading buffer and performing the gel electrophoresis as described above. Analysis was repeated three times and measurement error (as standard deviation) was calculated. Representative **Figure 3.14** shows band densities obtained with aforesaid siRNA concentrations as well as their relative band densities as compared to 50 pmoles concentration.

Calibration curve (**Figure 3.14, Figure 3.15** and **Table 3.3**) was generated by plotting relative band densities against concentration of siRNA. Curve was found to follow a linear equation y = 0.0207x + 0.034 with regression coefficient of 0.9977.

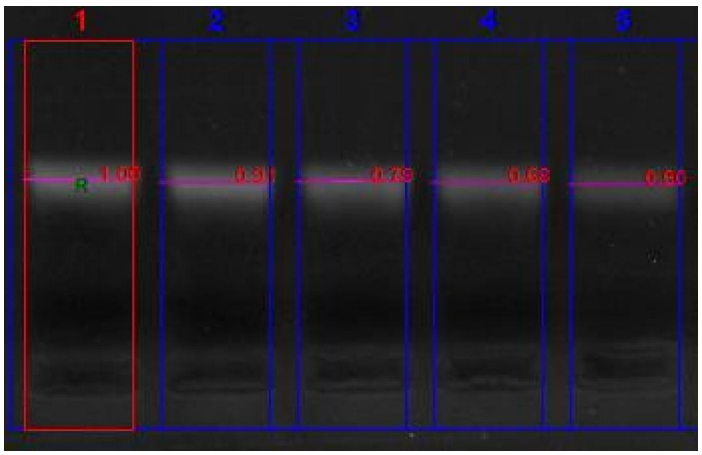


Figure 3.14 Gel electrophoresis band densities at different siRNA concentrations.
Lane 1-50 pmole, Lane 2- 45 pmole, Lane 3- 40 pmole, Lane 4- 35 pmole, Lane 5- 30 pmole.

Table 3.3 Relative band densities at different siRNA concentrations

Concentration of siRNA (pmole)	Relative Band Density	%Relative Standard Deviation
30	0.595±0.014	2.353
35	0.680±0.011	1.618
40	0.790±0.009	1.139
45	0.905±0.009	0.994
50	1.000	--

*Values are represented as mean±SD, n=3.

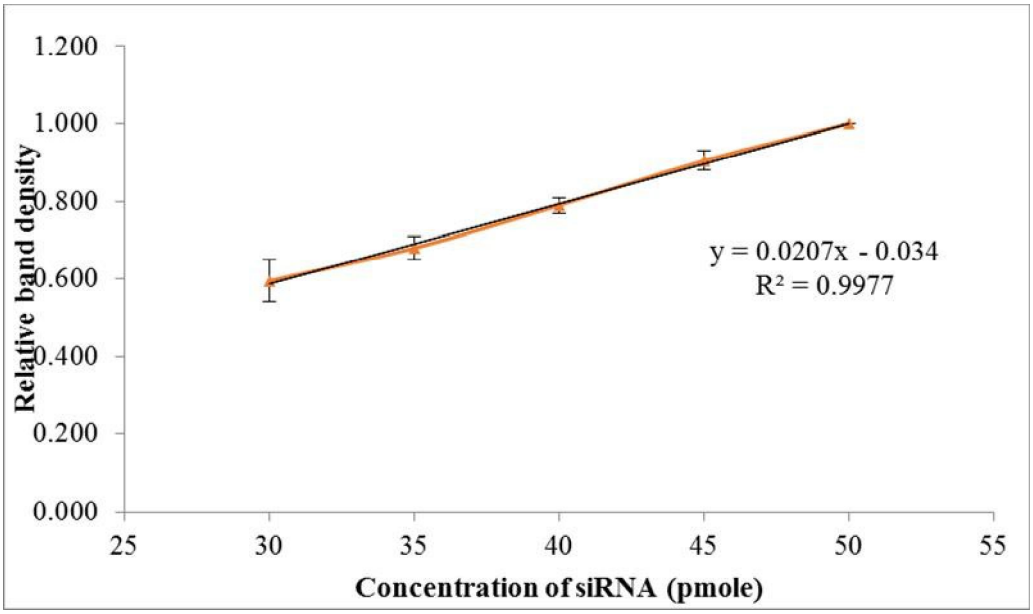


Figure 3.15 Calibration plot of siRNA gel retardation.

3.4.4 Accuracy and Precision of the Method

Accuracy and precision of gel electrophoresis assay method was determined using 50 pmol siRNA solution. Experiment was repeated for additional four times and relative band densities were determined using first band density as reference. From relative band densities % recovery and % relative standard deviation were calculated. **Figure 3.16** shows band densities of all repeated measurements.

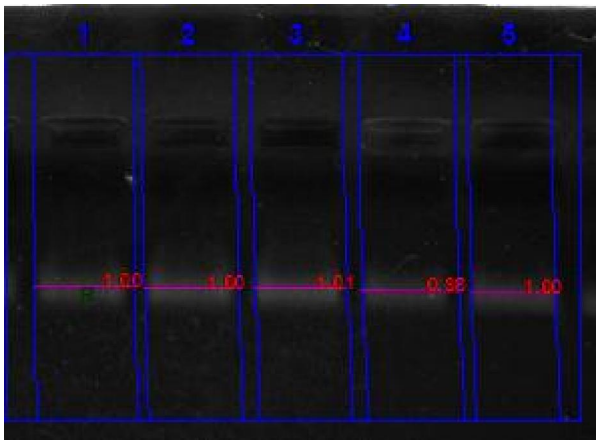


Figure 3.16 Accuracy and precision of gel electrophoresis for siRNA quantification.

% Recovery and % Relative Standard Deviation of the method were found to be 99.80±1.10% and 1.1% respectively which depict the accuracy and reproducibility of the method respectively. Therefore the proposed analytical method for quantification of siRNA was found to be reliable for routine estimations.

After the method of gel retardation was developed, selected siRNA was run against 10 bp ladder to confirm the siRNA length. siRNA migrated on the gel to an extent similar to the 20 bp band of the ladder (**Figure 3.17**). This confirmed the length of siRNA to be around 20 bp.

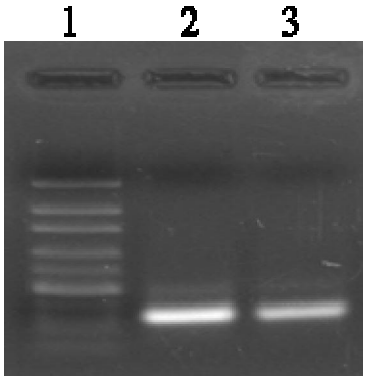


Figure 3.17 Detection of molecular weight of siRNA in comparison to DNA ladder. Lane 1: 10 bp ladder; Lane 2- Rat FGF2 siRNA; Lane 3: Bovine FGF2 siRNA

3.5 References

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