Chapter 3: siRNA Profiling and Analytical Method Development

3.1 siRNA Selection

Recent several studies show that neurotropins specifically brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF) may induce the airway hyper-responsiveness, which is an important characteristic of the asthma and chronic obstructive airway disease (COPD), by escalating sensory innervations. Neurotropins may also having tendency to induce inflammatory cells activation and migration which may further infiltrate airway bronchial mucosa and of numerous structural cells such as smooth muscle cells, epithelial cells, and fibroblasts(1). Selection of the siRNA (BDNF, a member of neurotrophin family) which is highly up regulated in the airway inflammatory conditions of bronchial asthma and in chronic obstructive airway conditions and also believed to be involved in the neurogenic inflammation and also involves in the smooth muscle contractility of the airways results in airway hyper reactivity and airway obstruction. Neurotropins also involves in crosstalk between nervous systems and immune system. So, by knocking down the expression of BDNF mRNA by siRNA can be promising therapeutic approach to treat obstructive airway disorders. The siRNA is ready available as customised and predesigned siRNA molecules 21 mer or 27 mer duplexes. SiRNA knockdown efficiency is based on the siRNA sequence such as GC content, design and nucleotides location at specific sites and siRNA duplex stability(2). All those important parameters and characteristics of predesigned siRNA were described in the subsequent section. SiRNA Purification is done by HPLC. Predesigned siRNA was procured as a ready to use HPLC grade duplex in the lyophilized form to be reconstituted in suitable quantity of nuclease free water.

3.2 siRNA Characterization

There are several thermodynamic and biophysical necessities such as GC content, Tm, MW, purity, which all confirms the siRNA functionality. The guanosine/cytosine (G+C) content of each duplex in most highly functional siRNAs ranges between 36% and 52%.

The relative stability and propensity to form internal hairpins can be assessed by the predicted melting temperatures (T_m). Moreover, Sequences with grater T_m values may favor the internal hairpin structures reducing their activity. In addition, duplexes showed $T_m < 60°C$ or predicted hairpin structures are better silencers(3, 4).

The characteristics of selected siRNA:

- ✓ **MW [g/mol]:** 13300
- ✓ **T_m [°C]:** 57.5
- ✓ **Purification:** HPLC
- ✓ **GC-Content [%]:** 42.8
- ✓ **Length**: 21-mer

The siRNA was purchased from Sigma Aldrich. The obtained siRNA as a dry pellet form was desalted, analyzed by the HPLC and diluted by nuclease free water to make required concentration of stock solution for the experimental work. The molecular weight of siRNA was 13,300 g/mole.

3.3 Analytical Method Development

It is required to confirm that siRNA be analytically evaluated for its integrity, quantity and its purity. There are some techniques for nucleotides estimation by analytically. siRNA quantity and purity has been determined using the spectroscopic technique due to absorptivity of nucleotides bases. While, integrity of the siRNA, which is indicator of the structural intactness can be determined by electrophoretic mobility assay like gel electrophoresis. Hence, it is necessities to develop systematic analytical method for siRNA profiling.

3.3.1 Material and Instruments

SiRNA duplex and Diethylpyrocarbonate (DEPC) were purchased from Sigma Aldrich, Bangalore; Tris buffer, Gel loading buffer, agarose was purchased from Himedia laboratories, Mumbai. Nuclease free water was prepared by treatment with DEPC, double distilled water was prepared in the laboratory, all the glassware's used were made RNase free by treating with DEPC treated water.

3.3.2 UV Spectrophotometric Analysis of siRNA by Nanodrop

(i) Quantification of siRNA Concentration:

Spectrometric technique has been the most common method of choice to estimate the amount of nucleic acids like DNA and RNA as it is simple and non-destructive technique

for the estimation of siRNA quantitatively which is based on absorbance of nucleotides at 260 and 280 nm selected as analysis constant and a baseline correction. For quantification of nucleic acid Beer-Lambert modified equation (1) is used to estimate sample concentrations.

$$c = (A * e)/b \quad \text{.....(1)}$$

Whereas, c = nucleic acid concentration (ng/μL), A = absorbance (AU), e = wavelength-dependent extinction coefficient (ng-cm/μL), b = path length (cm). The usually accepted extinction coefficients or constants for nucleic acids are as: Double-stranded DNA: 50 ng-cm/μL, Single-stranded DNA: 33 ng-cm/μL and RNA: 40 ng-cm/μL (5).

(ii) Purity of siRNA:

A260/280 Ratio

siRNA purity was confirmed by taking the ratio of A260/A280 and A260/A230. Nucleic acids and proteins have absorbance maxima at 260 and 280 nm, respectively. Moreover, theratio of absorbance at these wavelengths has been used as a measure of purity in both nucleic acid and protein extractions. Ratio of ~1.8 is usually accepted as “pure” for DNA; a ratio of ~2.0 is normally accepted as “pure” for RNA. It should be noted that these values are rule of thumb and the actual ratio depends on the nucleotide compositions of the nucleic acids.

A260/A230 Ratio

In the same way, absorbance at 230 nm is usually accepted as being the result of other contaminants; and hence the ratio of A260/ A230 is often also estimated. The 260/230 values for “pure” nucleic acid is often greater than the respective 260/280 values. While the expected 260/230 values are usually in the range of 2.0–2.2. in addition, residual chemical contamination from nucleic acids extraction procedures might result in an overestimation of the nucleic acid concentration and/or negatively influence downstream analysis(6).

(iii) Nanodrop spectrophotometer

Nanodrop spectrophotometer was used to estimate quantity and purity of siRNA duplex in the experiments as traditional absorption spectroscopy is comparatively insensitive and with conventional laboratory setup requires very less nucleotide concentration of at least 1 µg/ml. In order to fulfill this requirement, the traditional spectrophotometers cannot be used due to larger sample volumes for experiments (7, 8), NanoDrop measures absorbance at 320 nm to detect light-scattering components in the sample. The instrument software subtracts the reading at 320 nm wavelength from the 260nm, 280nm and 230nm values as background noise correction. Once siRNA was confirmed for its purity then a correlation curve was constructed for method verification using this siRNA duplex. siRNA stock solution was prepared in siRNA nuclease free water.

3.4 Nuclease Free Water Preparation:

Diethylpyrocarbonate (DEPC) is generally utilized reagent for preparation of NFW, buffer and other solutions. Diethylpyrocarbonate is an alkylating agent who reacts with -NH, -SH and -OH groups in RNases and other proteins, resulting in enzyme/nuclease inactivation. Hence, all the reagents and solutions used in RNA experiments were prepared in DEPC treated/Nuclease free water.

To prepare DEPC treated water, 1 ml of DEPC was mixed with 1 L doubled distilled water and stirred overnight at room temperature on magnetic stirrer. The dispersion prepared was then autoclaved at 121°C and 15 psi for 15 min to breakdown the residual DEPC and prepare NFW.

3.5 Method verification

Appropriate dilutions of stock solution (100 pmole/µl) of siRNA duplex were made with nuclease free water to perform method verification. Different concentration range of siRNA dilutions were prepared using nuclease free water. Absorbance of these solutions and stock solution were recorded at 260 nm on a NanoDrop UV spectrophotometer. The method was validated for analytical parameters.

3.6 Gel Electrophoresis

It is most important and pre-requisite to maintain the bioactivity and structural integrity of the siRNA till delivery site. siRNA is very unstable molecule and hydrolysis can lose its structural integrity. In compare to DNA, the presence of 2'-OH(hydroxyl) groups promote RNA hydrolysis under acidic and basic conditions and formation of smaller fragments of siRNA from the initial intact molecule(9). Hence, it is very important that the prepared formulation, as well as the developmental process be carried out in presence of a technique to detect the impact on the siRNA integrity.

Electrophoresis is commonly utilized for identification, quantification, and to access the purity of nucleic acid fragments. The gel electrophoresis works based on the principle that when charged molecules are placed in electric field, they travel towards oppositely charged electrode according to their charge. Phosphate backbone in the structure of the nucleic acid imparts considerable negative charge, so they migrate towards the anode in a "gel" matrix. For RNA applications the gel is composed of agarose at different concentrations of (0.5- 2%) for DNA and mRNA analysis. By melting the agarose and casting into the slab, gel can be prepared. During gelation process, agarose chains interact non-covalently to form a network of pores which decide molecular sieving characteristics of the gel.

Band detection of siRNA duplex can be done by different methods. The nucleic acid molecules can be visualized under UV light (>2500 µW/cm2) after staining with suitable dye e.g. Ethidium bromide (EtBr). Ethidium bromide is the most general dye used for this application and intercalate between the base pairs which results stabilization of the phenyl moiety and enhance in fluorescence of orange color corresponding to wavelength ~ 605 nm after excitation with UV (~305 nm). Sensitivity of the technique can be enhanced by using stronger fluorescing dyes such as SYBR® Gold and SYBR® Green II RNA gel stain. A UV transilluminator is typically used for this function. The light transmitted by fluorescing nucleotide can be detected after blocking the UV light using an orange filter.

Migration rate of the RNA in the gel network depends on its length or nucleotide numbers, shorter fragments moves faster than the longer RNA molecules. Fragmentation of the RNA molecules and loss of molecular weight results in lost in integrity of the molecules. This change can be detected through the gel electrophoresis and it is necessary tool for siRNA quality evaluation. Gel electrophoresis can give the information about siRNA quantity. The intact siRNA molecules give sharp bands when run on polyacrylamide gel. siRNA degraded partially, seems as smear due to low molecular weight and lack sharp bands. While siRNA degraded completely forms diffused faint band.

Protocol: Appropriate quantity of agarose was dissolved in 1x TBE buffer (Tris-Borate-EDTA), by heating and intermittent shaking to get a clear solution. Resultant Melted agarose solution cooled just above the pourable consistency. Meanwhile, gel tray for casting was prepared and tightly fastened at both the ends with tape to form a fluid-tight seal and comb was placed in the tray. To the cooled agarose, Ethidium bromide dye was (0.5 µg/mL) added before pouring. Gel loaded with ethidium bromide was casted with suitable height. Gel was allowed to set at 20°-25°C for 30 min. After solidification of the gel, the comb was removed cautiously, without damaging the wells, and tapes were taken off the edges to ensure conductive path in chamber. Gel was immerged in electrophoresis chamber (Genet Electrophoresis Powerpack, Banglore, India) with electrophoresis buffer (1x TBE buffer). Initially, minimum detectable siRNA quantity was estimated by starting from the range of lowest concentrations: 10-50pmole. After that the method for relative quantification based on densitometry was developed and verified by analysing increasing standard concentration of siRNA in the detectable range of 10, 20, 30, 40, 50, 75, 100, 200 pmole. The concentrations of siRNA were determined relative to reference band of 50 pmole. The method was validated for reproducibility by running 5 multiples and evaluating the Relative Standard Deviation.

3.7 Result and discussion:

siRNA selection and characterization.

siRNA sequence was selected by BLAST run of BDNF gene from NCBI sequence database to find most specific siRNA sequence and to diminish the off-target effects and provide definite gene silencing.The characterization details of the anti-BDNF siRNA are as fallows. Molecular weight of the siRNA duplex is 13,300 g/mol.

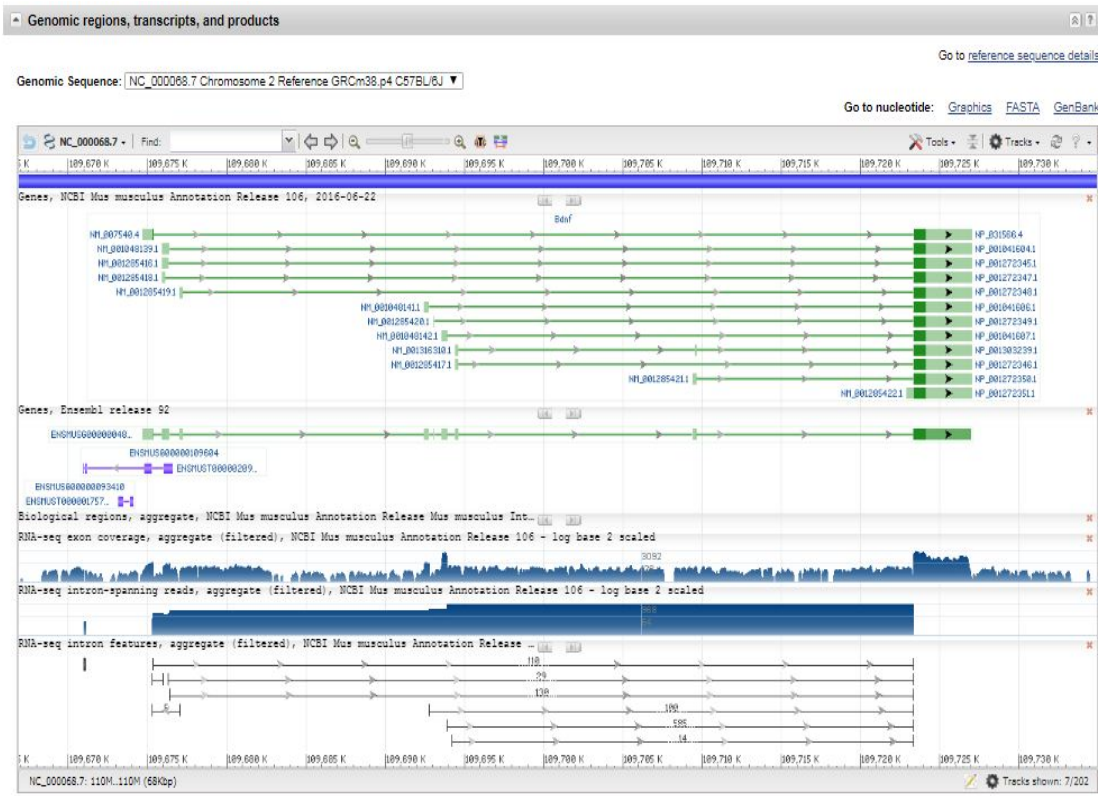

Figure 3.1: Genomic Regions details of Brain Derived Neurotrophic factor (Mus musculus (house mouse)

Brain Derived Neurotrophic Factor

Gene ID:12064

NCBI Reference Sequence: NC_000068.7

Sense strand Analysis

Strand sequences (5'→3'): **GAGACCAAGUGUAAUCCCA[dT][dT]**

T_m [°C]: 57.5

µg/OD:31.5

Purification: HPLC

Epsilon(1/mMcm):211.5

Length: 21-mer

Antisense strand analysis

Strand sequences (5'→3'):**UGGGAUUACACUUGGUCUC [dT][dT]**

T_m [°C]: 57.5

µg/OD:33.6

Purification: HPLC

Epsilon(1/mMcm):197

Length: 21-mer

Analytical Methods

Figure 3.2 demonstrates that the siRNA meets the purity requirements as the A260/A280 and A260/A230 ratio are within the acceptable range. 10 µl was the sample quantity requirement for experiments, which ensured minimum siRNA quantity.

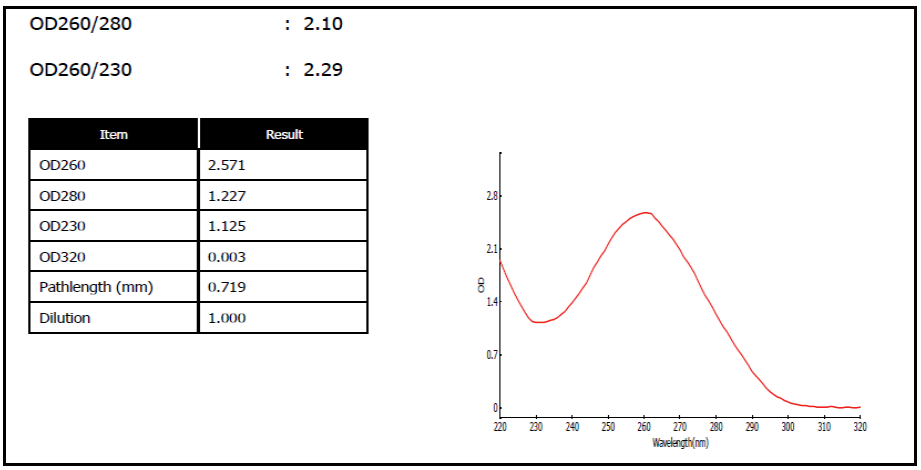

Figure 3.2: SiRNA concentration estimation by Nanodrop UV Spectrophotometer

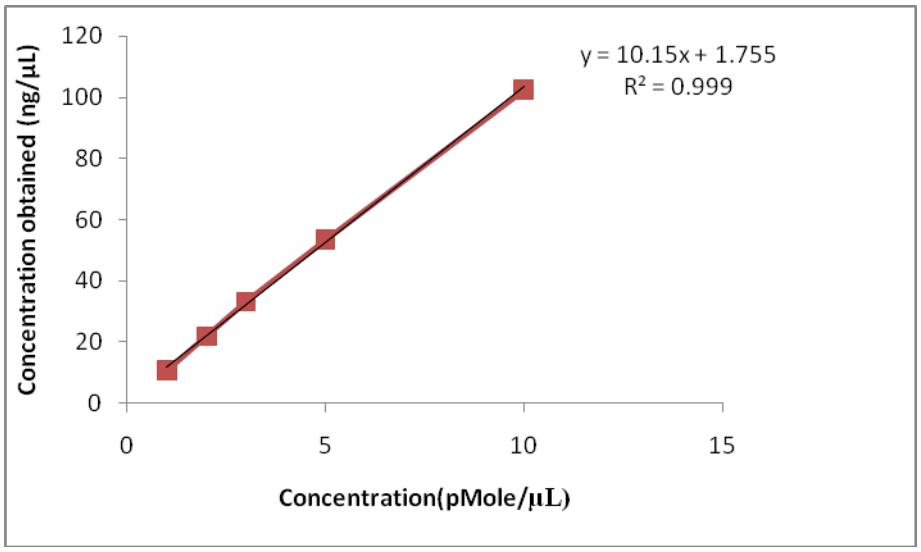

Figure 3.3: known concentration of siRNA Vs NanoDrop concentration correlation

Figure 3.3 shows the correlation between prepared and obtained concentration of siRNA demonstrated that the method can be used for determination of concentrations from minimum of 1pmole/µl to avoid instrument noise up to 10 pmole/µl to avoid detector saturation as the absorbance was in the range of 0.2 to 1.2 (Table 3.1). This was linearity range for analysis of siRNA by using NanoDrop.

Table 3.1 siRNA concentration obtained at 260 nm

Concentration of siRNA (pmole/µL)	Obtained Concentration (ng/ µL)	Standard Deviation	%Relative Standard Deviation
1	10.77	0.089	0.829
2	21.93	0.105	0.481
3	33.02	0.123	0.369
5	53.8	0.137	0.255
10	102.72	0.164	0.159

The verification of method involved validation for accuracy and precision. Accuracy, defined as closeness of the true and measured readings values of a sample. It was determined from making repeat measurements of three samples concentration. From the calibration plot three different quality control (QC) levels of siRNA concentrations were selected [lower quality control sample (LQC) = 1 pmole/µl, medium quality control sample (MQC) = 5pmole/µl, and higher quality control sample (HQC) = 10 pmole/µl]. The samples were prepared from stock solution independently and analysed in triplicate. Accuracy was quantified by calculating mean percentage recovery

Repeatability was checked by measuring different QC levels as mentioned in accuracy. To determine intermediate precision, inter and intra-day variations were studied. Inter–day variation study was carried out for 3 days. The calculated % RSD of the concentrations was taken as precision.

Table 3.2 and 3.3 depict results of accuracy, intraday and inter-day precision of the method respectively. It can be seen that % recovery was found to be in the range of 98.0% to 100.0% which was within the acceptable range. Further, the % Relative standard deviation (RSD)values were less than 2% in the precision study and are within the acceptable range as per the requirements of ICH guidelines. Therefore, the method

can be used for determination of quantity of siRNA in the sample down to a concentration of 1pmole/µl.

Table 3.2: Results of accuracy measurements

Concentration (pmole/µL)	Obtained Concentration (ng/µL)	Standard Deviation (SD)	%Recovery
1	10.61	0.084	98.51
5	53.72	0.134	99.85
10	102.64	0.186	99.92

All the measurements were taken in triplicates i.e. n= 3

Table 3.3: Intraday and Interday precision of the method

	Observed concentration		%Relative Standard Deviation	
Concentration (pmole/µL)	Intraday precision*	Interday Precision*	Intraday precision	Interday Precision
1	10.61± 0.084	10.57± 0.071	0.981	1.034
5	53.72± 0.134	53.65± 0.108	0.322	0.411
10	102.64± 0.186	102.69±0.205	0.198	0.206

*Values are represented as Mean± SD, n= 3

Gel electrophoresis

This technique was proposed to study the siRNA integrity and the complexation efficiency of the developed non-viral vectors of siRNA. As the vectors being studied had pH dependent ionization, the electrophoresis buffer pH was selected to be 7.4 as it is physiologically relevant condition and the vectors being studied had a ionisation based on pH. The pH adjustment of TBE buffer was important step in the analysis. The pH of unadjusted 1X TBE buffer is around 8.0. Therefore, it has to be acidified with suitable acid to decrease the pH to 7.4. pH adjusted with the sodium

acetate which having lower conductivity of the acetate ions compared to the hydrochloric acid as acid have high conductivity chloride ions so result in band dragging of the siRNA under electrophoresis voltage.

Table: 3.4 Optimized parameters for Agarose gel electrophoresis

Parameters	Optimized condition
Detectable siRNA	30 pmole
Tank buffer	TBE, 7.4 pH
Voltage,	100 V,
Vehicles for siRNA	sodium acetate
Agarose Gel	2% w/w
Run time	40-45 min

Quantification of siRNA:

A densitometry technique was developed for quantification of siRNA on agarose gel. Initially, the lowest detectable quantity of siRNA with the optimized gel electrophoresis conditions was determined so further calibration range can be estimated and evaluation can be performed in minimum siRNA quantity. For this purpose, siRNA at increasing concentration of 10, 20, 30, 40, 50 pmole in nuclease free water and run for electrophoresis.

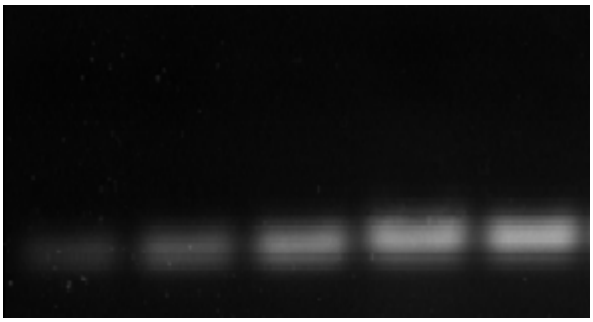

Figure 3.4: Estimation of minimum quantity of siRNA by gel retardation assay
Lane (L→R) Lane 1: 10pmole; Lane 2:20 pmole; Lane 3: 30 pmole; Lane 4:40pmole. Lane 5:50 pmole.

To enable quantification by densitometry, the relative quantification method was developed and partially validated for analytical parameters like accuracy and precision. The siRNA concentration of 30,35,40,45 and 50pmole was run on gel with gel loading buffer and run on 2% agarose gel at 100 V. siRNA was detected with ethidium bromide and visualized on UV transilluminator Gel Doc™ XR$^+$ Imaging System (BioRad, USA). The band density at 50pmolewas taken as reference i.e. 1 and remaining bands were quantified relative to it. The Image lab (version 5.2.1) software was used for densitometry analysis. Fig.3.5demonstrates that siRNA concentration of >30 pmole is detectable with good visual assessment.

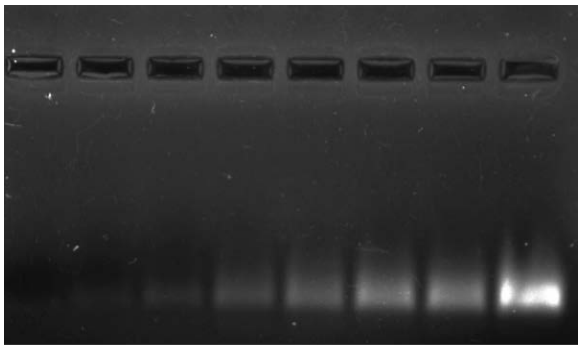

Figure:3.5 Determination of Quantifiable Range of siRNA

Lane (L→R) (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)

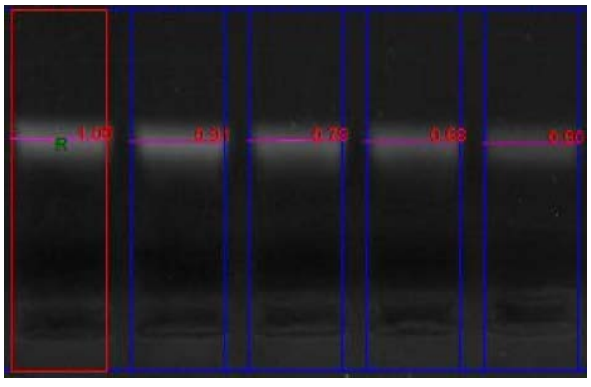

Figure 3.6: Gel electrophoresis band densities at different siRNA concentrations

Lane L→R (Lane 1: 50 pmole, Lane 2:45 pmole,Lane 3:40 pmole, Lane 4: 35 pmole, Lane 5:30 pmole)

Figure 3.6 demonstrates the band density for different concentrations of siRNA relative to 50pmole internal standard. The Table 3.5 shows correlation between concentration and obtained band density. The RSD values for all the densitometry analysis were < 3.0%. Further, the correlation curve between concentration and relative band density was prepared (Fig.3.7). It was observed that there was a perfect correlation with R^2value of 0.998. Therefore, the method is accurate for estimations with use of internal standard in analysis.

Table 3.5: Relative band densities at different siRNA concentrations

Concentration of siRNA (pmole)	Relative Band Density	%RSD
30	0.58±0.011	2.41
35	0.67±0.012	1.82
40	0.78±0.01	1.52
45	0.89±0.010	1.08
50	1.000±0.000	-

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

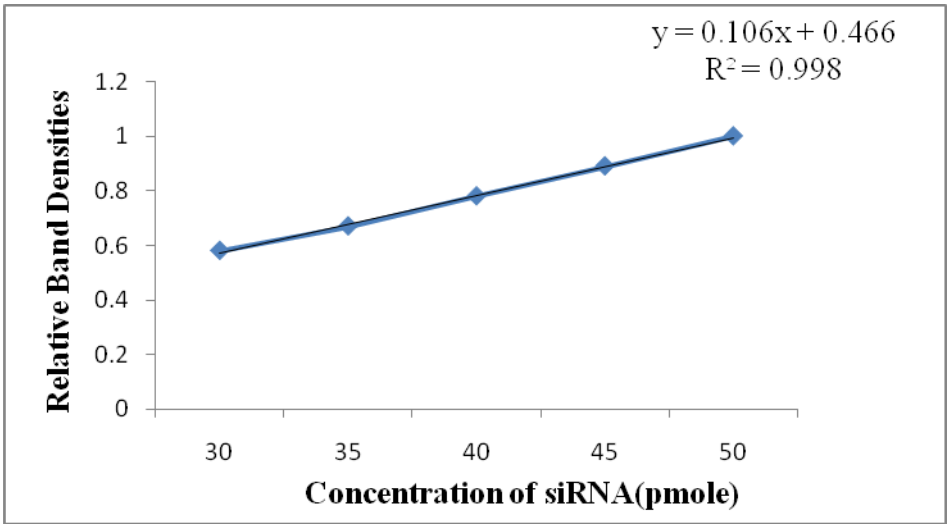

Figure 3.7: Calibration curve of siRNA gel retardation

Accuracy and Precision of the Method

The method was partially validated for analytical parameter of accuracy and precision. The analytical reproducibility was assessed by running the test concentration of 50pmole in five replicates and using the relative densitometry to determine the concentration of each run (Fig.3.8).

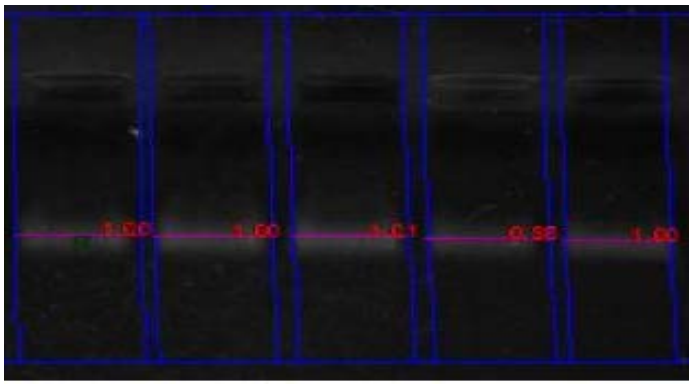

Figure 3.8: Accuracy and precision of Gel Electrophoresis Method for siRNA Quantification. Lane L→R (Lane 1to 5: 50 pmole)

The method was analysed for accuracy and precision parameters. The accuracy was analysed in terms of % recovery and % bias. The % recovery for the method was 98.80 ±0.023 which was within the acceptable limits. The precision was analysed by % Relative standard deviation. The % RSD was 1.45 for 5 multiples which was also within the acceptable limits. Therefore, it can be concluded that this method can be used for reliable quantification of siRNA concentrations in the experiments.

Hence, the procured Brain derived neurotrophic factor (BDNF) siRNA was found to meet the purity requirements as tested by absorption ratios-based estimation by nanodrop spectrophotometer. The agarose gel electrophoresis technique was suitable and highly useful for evaluation of siRNA quality and quantity. The gel electrophoresis technique was developed to obtain condensed bands of the samples to be tested to ensure reproducibility and accuracy of the findings. The technique was accurate and precise within the analytical considerations.

3.8 References:

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