

**Chapter 3**  
**Analytical method**  
**development**

### 3 Analytical Method Development

#### 3.1 Selection of siRNA

siRNA targeting NRG1 was procured from Sigma Aldrich. The characteristics of the procured siRNA can be depicted as follows:

- Sense strand sequence: 5'-CUCAUAAAGUGUGCGGAGA[dT][dT]-3'
- Anti-sense strand sequence: 5'-UCUCCGCACACUUUAUGAG[dT][dT]-3'
- MW [g/mol]: 13300 g/mol
- T<sub>m</sub> [°C]: 56.5 °C
- GC-Content [%]: 42.8%
- Length: 21 base-pair
- Purification: Desalted

#### 3.2 UV spectrophotometric analysis

siRNA was quantified using UV spectrophotometric method [1, 2]

##### 3.2.1 Preparation of siRNA stock solution

For preparing siRNA solution nuclease free water is required viz. prepared by adding diethyl pyrocarbonate (DEPC) in double distilled water such that final concentration of DEPC is 0.1% (i.e. 1 ml DEPC is added in 1 l distilled water). This was then sterilized by autoclaving at 15 PSI for 15 min. 500 µl of the resultant DEPC treated water (nuclease free water) was added to 50 nmole lyophilized siRNA pellet and mixed gently to obtain stock solution.

##### 3.2.2 Determination of purity of siRNA sample

siRNA purity was determined using BioSpec-nano micro volume UV-Vis spectrophotometer (Shimadzu, Japan) by measuring OD of solution at 230, 260 and 280 nm wavelength. Then, ratio of A<sub>260</sub>/A<sub>280</sub> was determined which was used as parameter indicating purity of prepared sample. The ideal value of it should be in range of 1.8-2.2 [3] indicating highly pure sample. Moreover, A<sub>260</sub>/A<sub>230</sub> was used as secondary measure for nucleic acid purity. Expected values of it should be 2.0-2.2 and higher than A<sub>260</sub>/A<sub>280</sub> [4-6]. Lower values indicate presence of contaminants like EDTA, carbohydrates, phenol etc. which have absorbance at 230 nm. The average value of A<sub>260</sub>/A<sub>280</sub> was 2.03 ± 0.11 while that for A<sub>260</sub>/A<sub>230</sub> was 2.18 ± 0.12 which were

within the expected standard limits for purity and thus the siRNA solutions and samples prepared were considered pure.

### 3.2.3 Quantification of siRNA

Quantification of siRNA was performed by UV spectroscopy method at 260 nm. All the apparatus were washed with DEPC treated water to remove and inactivate the nucleases. Then, stock solution of siRNA was prepared by dissolving required quantity of siRNA in nuclease free water to get final concentration of 100 pmole. This stock solution was appropriately diluted using nuclease free water to get desired concentration in range of 5-50 pmole. Later, using BioSpec-nano micro volume UV-Vis spectrophotometer, concentration in ng/ $\mu$ l was determined and compared with the original sample concentration. Average concentration of the siRNA solutions obtained along with their standard deviation is depicted in Table 3. 1. This was then plotted as calibration plot of actual concentration vs observed concentration to determine linearity and reproducibility of the obtained results and calibration curve is shown in Figure 3. 1. Calibration plot obtained was linear expressed by equation  $y = 1.3212x + 0.683$  with regression coefficient value of 0.9991. Although, estimation at 260 nm can accurately predict siRNA concentration present in the solution, determination at 280 and 230 nm is equally important to estimate purity of sample as detection at 280 nm can predict protein contamination in siRNA sample while detection at 230 nm predicts EDTA, carbohydrates and phenolic contaminants.

Table 3. 1: Concentration of siRNA at 260 nm

Actual Concentration		Observed concentration* (ng/ $\mu$ l)
pmole	ng/ $\mu$ l	
5	6.65	7.83 $\pm$ 0.09
10	13.3	14.05 $\pm$ 0.13
15	19.95	19.86 $\pm$ 0.12
20	26.6	26.88 $\pm$ 0.17
25	33.25	33.51 $\pm$ 0.30
30	39.9	40.96 $\pm$ 0.42
40	53.2	52.63 $\pm$ 0.62
50	66.5	67.38 $\pm$ 0.93

\*Experiment was performed in triplicate

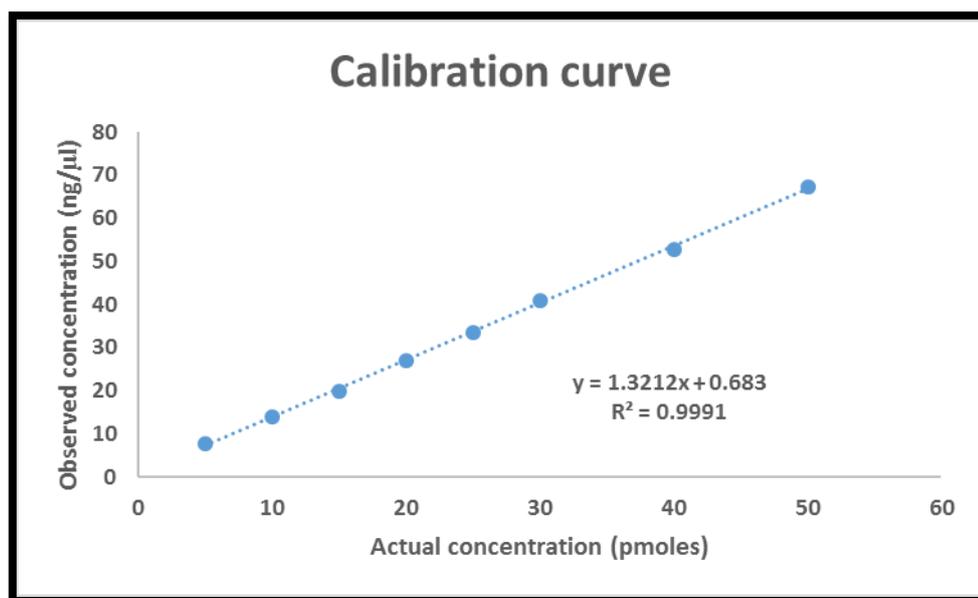


Figure 3. 1: Calibration curve of siRNA comparing observed and actual concentration

### 3.2.4 Accuracy and Precision

These parameters were determined by estimating % recovery and relative standard deviation (RSD). siRNA standard solutions of 15, 20, 40 and 50 pmole concentration were prepared in nuclease free water and were analyzed for absorbance by BioSpec-nano micro volume UV-Vis spectrophotometer. The accuracy of method was then determined by measuring % recovery of the known concentrations while precision was determined by repeated absorbance measurement at different time intervals and finding RSD. All the measurements were performed in triplicate and results are shown in Table 3. 2 and Table 3. 3. As evident from the results, % recovery was found within 98-102% while %RSD was less than 2% meeting the acceptance criteria as per the ICH guidelines.

Table 3. 2: Accuracy determination for UV spectrophotometric method

Concentration (pmole)	Actual concentration (ng/μl)	Observed concentration* (ng/μl)	Standard deviation	%Recovery
15	19.95	19.81	0.31	99.30
20	26.6	26.31	0.29	98.91
40	53.2	52.88	0.89	99.40
50	66.5	67.03	1.04	100.80

\*Experiment was performed in triplicate

Table 3. 3: Interday and Intraday precision of the UV spectroscopic method

Concentration (pmole)	Intraday precision		Interday precision	
	Observed concentration* (ng/ $\mu$ l)	%RSD	Observed concentration* (ng/ $\mu$ l)	%RSD
15	19.81 $\pm$ 0.31	1.57	19.98 $\pm$ 0.27	1.37
20	26.31 $\pm$ 0.29	1.09	26.54 $\pm$ 0.42	1.57
40	52.88 $\pm$ 0.89	1.69	53.12 $\pm$ 0.22	0.42
50	67.03 $\pm$ 1.04	1.55	66.95 $\pm$ 1.12	1.67

\*Experiment was performed in triplicate

### 3.3 Agarose gel electrophoresis

This technique employs principle of gel retardation of siRNA complexed with vectors and thus was used for relative quantification of free siRNA. This was in-turn used to estimate conjugation efficiency of siRNA with non-viral vectors.

Agarose gel electrophoresis separates mixture of nucleic acids based on their size. In this technique, when electric field is applied, negatively charged nucleic acids (DNA or RNA) migrate from negative electrode to positive electrode. This migration of nucleic acid is size dependent i.e. smaller nucleic acids migrate faster while larger molecules migrate slower through gel pores [7]. Thus, free siRNA would move faster and travels more distance as compared to siRNA complexed with polymeric vectors and therefore can be detected differentially. The quantity of free siRNA can provide idea regarding quantity of conjugated siRNA and in-turn conjugation efficiency of siRNA with polymeric vectors

The migrated siRNA can be detected by addition of ethidium bromide in agarose gel as well as siRNA samples. Ethidium bromide is nucleic acid intercalator, which can intercalate between the base pairs of nucleic acid and give fluorescence when observed under UV light [8].

#### 3.3.1 Procedure

1% agarose gel was prepared by dispersing the required quantity of agarose in 1x TAE (Tris-acetate-EDTA) buffer. The mixture was heated in heating mantle, with continuously shaking the flask while boiling, to dissolve the agarose. Care should be taken to avoid excessive or localized heating of the agarose gel which may give faulty results. The gel tray was securely sealed at the ends by fixing it in the gel casting tray.

The comb was placed over the gel tray. Later, when agarose was sufficiently cooled, ethidium bromide (0.5  $\mu\text{g/ml}$ ) was added, stirred and the resulting gel was poured into the gel tray to a depth of 48 mm. The gel was allowed to set at 20  $^{\circ}\text{C}$  for 30 min followed by refrigeration for further 15 min for complete solidification of gel. The comb was removed from the solidified gel carefully. The gel was then transferred to the electrophoresis chamber and submerged into the electrophoresis buffer (1x TAE buffer).

### 3.3.2 Determination of quantifiable range of siRNA for gel retardation assay

Before quantifying siRNA, initially siRNA solutions of different concentrations (5, 10, 25, 50, 100, 250 and 500 pmole) were prepared. Solutions were mixed with sufficient quantity of gel loading buffer (glycerol 30 %w/v + bromophenol blue 0.25 %w/v) by using a vortex mixture in 0.5 ml microcentrifuge tubes. siRNA samples (10  $\mu\text{l}$ ) were loaded in the wells and electrophoresis was carried out at 100 V/cm. The gel was removed and siRNA in the agarose gel was visualized under UV light using GelDoc<sup>TM</sup> XR+ Imaging System (Biorad, USA).

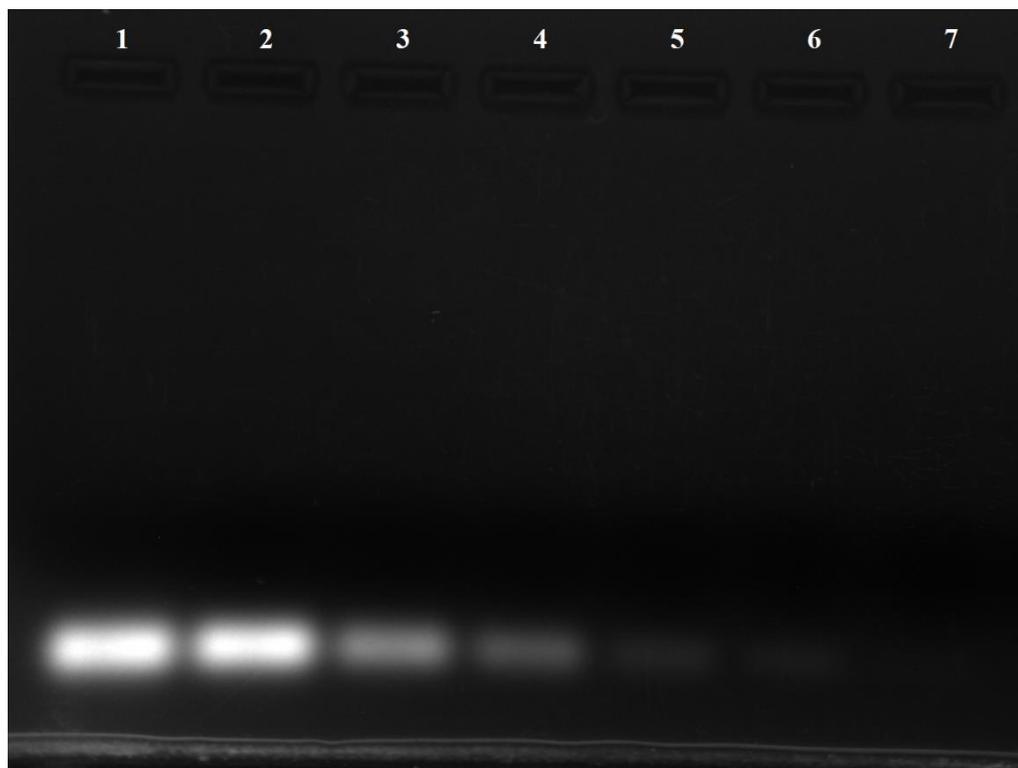


Figure 3. 2: Detection of quantifiable range of siRNA by agarose gel electrophoresis

Lane 1: 500 pmole; lane 2: 250 pmole; lane 3: 100 pmole; lane 4: 50 pmole;  
lane 5: 25 pmole; lane 6: 10 pmole; lane 7: 5 pmole

As shown in Figure 3. 2 it was found that minimum quantity of siRNA that could be detected by agarose gel electrophoresis was 25 pmole while bands of lower siRNA concentrations were not sufficiently intense to be quantified under similar conditions.

### 3.3.3 siRNA calibration curve

After determining the minimum quantifiable siRNA concentration, calibration curve of siRNA using agarose gel electrophoresis was plotted. For that, siRNA solutions of different concentrations (25, 50, 60, 70, 80, 90 and 100 pmole) were prepared. The prepared siRNA solutions were mixed with sufficient quantity of gel loading buffer (glycerol 30 %w/v + bromophenol blue 0.25 %w/v) by using a vortex mixture in 0.5 ml microcentrifuge tubes. siRNA samples (10  $\mu$ l) were loaded in the wells and electrophoresis was carried out at 100 V/cm. The gel was removed and siRNA in agarose gel was visualized under UV light using GelDoc™ XR+ Imaging System. The calibration curve was prepared by plotting siRNA absolute quantity vs band volume (x1000). The analysis was performed in triplicate to minimize errors and improve precision of the analytical method.



Figure 3. 3: Agarose gel electrophoresis band density at various siRNA concentrations

Lane 1: 25 pmole; lane 2: 50 pmole; lane 3: 60 pmole; lane 4: 70 pmole;

lane 5: 80 pmole; lane 6: 90 pmole; lane 7: 100 pmole

Figure 3. 3 presents the representative agarose gel electrophoresis of siRNA at varied concentration from which band volume was calculated and obtained results are presented in Table 3. 4. Calibration curve of siRNA absolute quantity vs band volume (x1000) was plotted as shown in Figure 3. 4.

Table 3. 4: Band volume of siRNA at different concentrations

Concentration (pmole)	Band volume* (x1000)	%RSD
25	1669.14 ± 23.185	1.389
50	5418.501 ± 55.567	1.026
60	7277.224 ± 36.591	0.503
70	8574.363 ± 68.962	0.804
80	10121.888 ± 87.739	0.867
90	11506.922 ± 144.935	1.260
100	13537.782 ± 128.468	0.949

\*Experiment was performed in triplicate

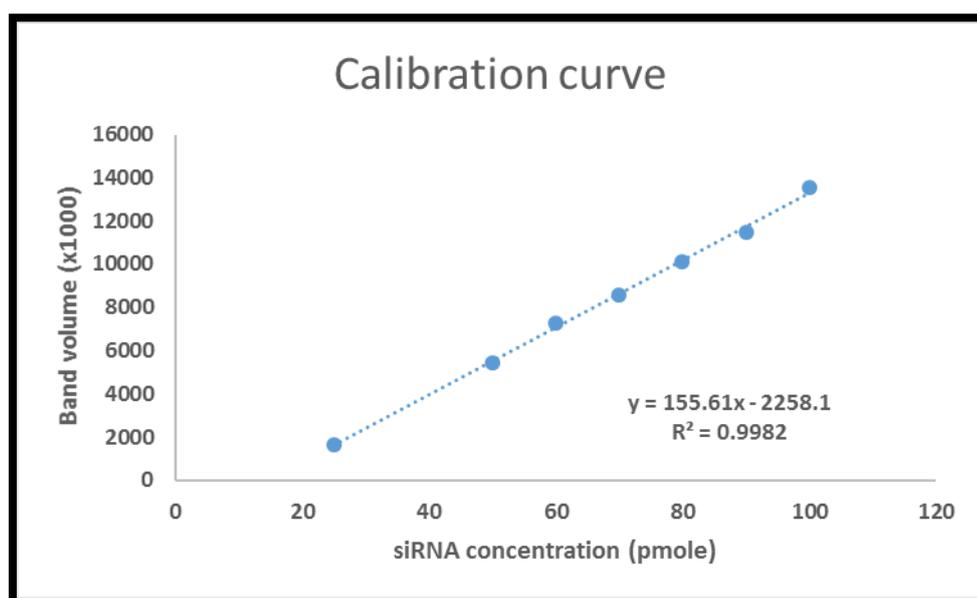


Figure 3. 4: Calibration curve of siRNA gel electrophoresis

### 3.3.4 Accuracy and precision of method

Accuracy and precision of gel electrophoresis assay was determined by loading 100 pmole of siRNA solution in 8 different wells. The gel obtained (Figure 3. 5) was viewed using GelDoc™ XR+ Imaging System and band volume of all the bands was calculated which are presented in Table 3. 5. Average band volume of 8 measurements

was compared with band volume of 100 pmole siRNA concentration used in calibration curve and % recovery was determined. Precision was determined by comparing all the 8 measurements and calculating %RSD.

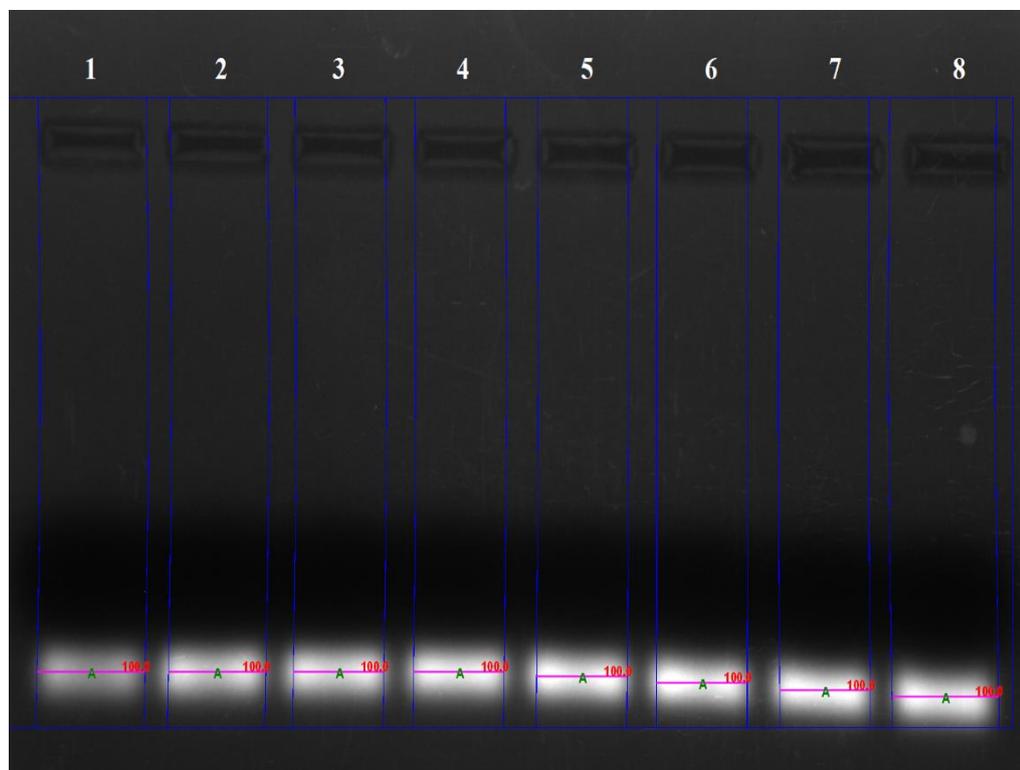


Figure 3. 5: Accuracy and precision of agarose gel electrophoresis assay

Table 3. 5: Band volume (x1000) of 100 pmole siRNA

Band No	Band volume (x1000)	% recovery
1	13496.52	99.69521
2	13523.087	99.89145
3	13443.504	99.30359
4	13657.084	100.8813
5	13529.29	99.93727
6	13573.47	100.2636
7	13588.762	100.3766
8	13450.057	99.352
<b>Mean</b>	13532.722	99.96
<b>SD</b>	72.186	0.53
<b>%RSD</b>	0.533	0.533

% recovery and % RSD of the assay were found to be  $99.96 \pm 0.53$  and 0.533 respectively which depicts accuracy and precision of the siRNA quantification assay. Thus, this analytical method was found to be reliable and reproducible for siRNA quantification under similar conditions

### 3.4 References

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