Method Evaluation for RNA Purity Analysis Using CE-LIF Technology

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Introduction

CE has been demonstrated to be one of the most powerful techniques for analysis of a wide variety of molecules. The PA800 Plus Pharmaceutical Analysis System from SCIEX is an outstanding instrument, specifically designed and optimized for capillary gel electrophoresis (CGE) separation, which is widely used for separation of biologics such as protein, peptide, nucleic acids, etc.

Comparing to traditional slab-gel based electrophoresis methods, CGE offers superior resolution, shorter analysis time, automated operation and exceptional sensitivity when combined with a laser induced fluorescence detector. Compared to chip-based CE systems, the PA800 Plus provides open chemistry, which enables flexibility for method modification and optimization to generate optimal results for each specific project.

Recent advances in gene therapy research have gained promise in the utility of gene therapeutics compounds. The ability to quantify RNA purity and quality is critical to ensure the safety and efficacy of these molecules. In this study, a CE-LIF fast separation method which was used to evaluate total RNA quality[1] was optimized to achieve higher resolution; and the method was then evaluated for RNA purity determination. The resolution of this method was optimized for RNA size ranging from 200 bases to 6583 bases and evaluated by spiking a 1.2 kb positive RNA marker into several commercially available RNA ladders. A calibration curve was generated from the RNA ladders which could then be used to estimate the size of unknown sample peaks. In addition, assay repeatability, linearity, LOQ, LOD were also evaluated in this study. The optimized method could be used as an RNA platform analytical method for RNA analysis or further modified to suit more specific user criteria.

Key Features

- Baseline resolution of RNA ladders from 0.2 kb to 6.5 kb
- Capability of RNA size estimation of unknown samples using calibration curve of RNA ladders

The PA 800 Plus Pharmaceutical Analysis System

- Good repeatability with %RSD of MT within 0.1% and %RSD of %CPA within 5%
- Excellent Linearity with R2=0.996 of detection response vs concentration
- LOQ = 0.33 ng/mL and LOD = 0.081 ng/mL

Materials and Methods

Urea was obtained from ThermoFisher Scientific (PN 29700, Waltham, MA). Polyvinylpyrrolidone was obtained from Sigma-Aldrich (PN 437190, St Louis, MO). TBE Buffer, 10X, Molecular Biology Grade was obtained from Sigma-Aldrich (PN 574795, St Louis, MO). SYBR™ Green II RNA Gel Stain, 10,000X concentrate in DMSO was obtained from ThermoFisher Scientific (PN S-Waltham, MA), Ladder of Nine RNA Transcripts 281-6583 bases was obtained from Promega (PN G3191, Fitchburg, WI), 1.2 kb Kanamycin Positive Control RNA was obtained from Promega (PN C1381, Fitchburg, WI).

1%PVP (1.3 MDa) in 1X TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) with 4 M urea and 50,000x dilution or 0.002% SYBR green dye was used as separation buffer.

RNA ladders and 1.2 k RNA marker were diluted in DDI water, denatured for 5 minutes at 65° C and cooled down on ice

for 5 minutes before loading. Two RNA ladders were used in this study. One is the Thermo RNA 6000 Ladder of six RNA transcripts with lengths of 0.2, 0.5, 1.0, 2.0, 4.0, and 6.0 kb. The other one is the Promega RNA marker which consist of aladder of nine RNA transcripts of 281, 623, 955, 1,383, 1,908, 2,604, 3,638, 4,981 and 6,583 bases. Both ladders were diluted to 25 µg/mL and spiked with the proper amount of the 1.2 kb marker, respectively.

All experiments were performed on PA800 plus Pharmaceutical Analysis System from SCIEX.

EZ cartridge pre-assembled with bare fused-silica capillary (50 µm I.D., 30 cm total length, 20 cm effective length) was purchased from SCIEX (PN A55625, Framingham, MA).

Samples were introduced into the inlet of the capillary either electrokinetically at -5kV for 3s. Separations were performed using reversed polarity with 300V/cm electrical field at 25° C. Samples Tray were kept at 4° C to minimize RNA degradation and renaturation. LIF detector was configured with a 488-nm laser with an emission filter of 520 nm.

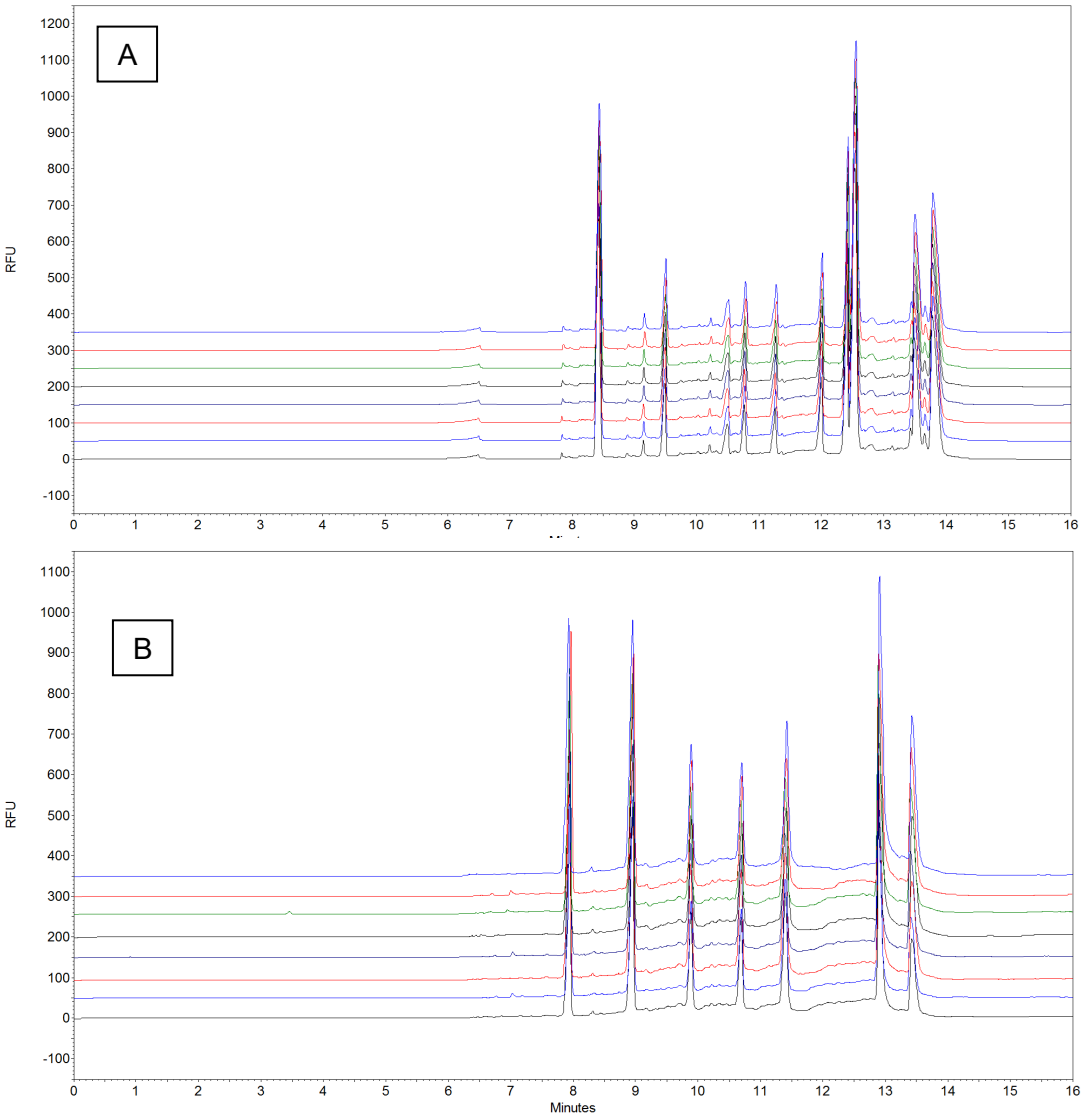

Figure 1. a) The overlay of the eight consecutive analyses of the Promega RNA ladder with spiked in 1.2 kb marker. b) The overlay of the eight consecutive analyses of the Thermo RNA 6000 ladder with spiked in 1.2 kb marker.

Results and Discussion

Method optimization

Both the Thermo RNA6000 ladder with spiked in 1.2 kb marker and Promega ladder with spiked in 1.2 kb marker were used for method optimization as the Thermo RNA 6000 ladder has less impurity peaks and Promega ladder has more markers of different sizes in the mixture. Different separation voltages and separation temperature were compared to find the optimal resolution and peak shape. Figure 2 compares the separation of Thermo RNA6000 ladder (spiked in 1.2 kb marker) using 9 different separation voltages (25 kV, 20 kV, 15 kV, 12 kV, 10 kV, 8 kV, 6 kV and 5 kV) and a capillary temperature of 25° C. All The 6 markers in the RNA 6000 ladder and a spiked in 1.2kb RNA molecule were baseline resolved using 6 kV and 5kV of separation voltage at 25° C. Figure 3 shows the similar study using RNA ladder from Promega, which has 9 markers, including a spiked in 1.2 kb molecule. All 9 RNA markers (281 bases, 623 bases, 955 bases, 1.2 kb,1383 bases, 1908 bases, 2604 bases, 3638 bases, 4981 bases and 6583 bases) were baseline resolved using 8 kV and 6 kV of separation voltage at 25° C. A separation voltage of 6 kV and capillary temperate of 25° C provided the best resolution and peak shape for these two different RNA markers.

Similar optimization work was performed using capillary cartridge temperatures of 15° C, 20° C, 30° C, 35° C and 40° C (data not shown). Of all the cartridge temperature and electric field conditions tested, 6 kV (200 v/cm) at 25° C was considered the optimized condition when comparing factors such as analysis time, resolution, peak shape, and stability of RNA sample solutions. Additional work was done to evaluate other parameters such as 2% PVP in the separation buffer, injection method (pressure injection for 3s at 0.5 psi, electrokinetic injection for 5s at 2 kV and 3s at 5 kV) and capillary length (10 cm and 20 cm effective length options provided by EZ cartridge). The following optimized method conditions were determined: separation buffer containing 1% PVP, electrokinetic injection for 3s at 5 kV, 20 cm effective length capillary and separation with 200 V/cm electrical field (6 kV) at 25° C. These method parameters were used in the evaluation study.

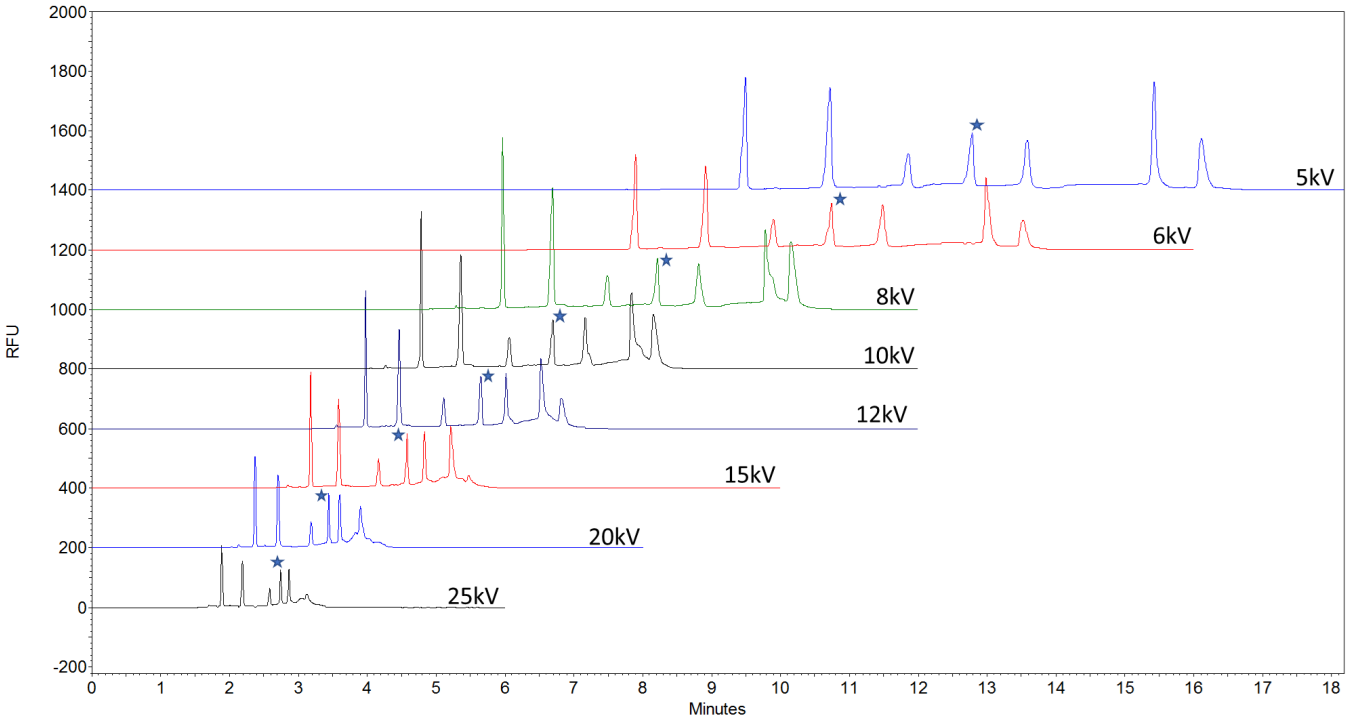

Figure 2. Overlay traces of RNA 6000 ladder with spiked in 1.2 kb marker separated using different electric field under 25° C. The 6 RNA markers in each trace are 0.2 kb, 0.5 kb, 1.0 kb, 2.0 kb, 4.0 kb and 6.0 kb. The peak with star mark is the spiked in 1.2 kb molecule. The separation voltages used from bottom to top traces were 25 kV, 20 kV, 15 kV, 12 kV, 10 kV, 8 kV, 6 kV and 5 kV.

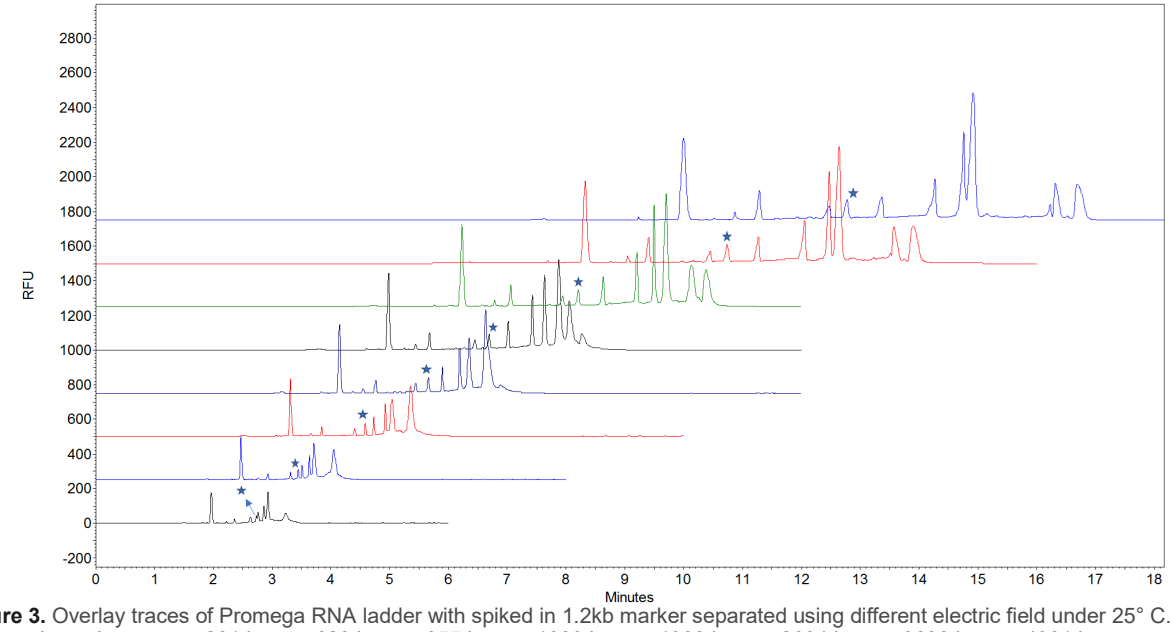

Figure 3. Overlay traces of Promega RNA ladder with spiked in 1.2kb marker separated using different electric field under 25° C. The 9 RNA markers in each trace are 281 bases, 623 bases, 955 bases, 1383 bases, 1908 bases, 2604 bases, 3638 bases, 4981 bases and 6583 bases. The peak with star mark is the spiked in1.2 kb molecule. The separation voltages used from bottom to top traces were -25 kV, -20 kV, -15 kV, -12 kV, 10 kV, 8 kV, 6 kV and 5 kV.

Calibration Curve

Figures 4 and 5 illustrate the typical electropherograms of the analysis of the RNA6000 ladder and Promega RNA Ladder using the optimized method conditions. Figures 6 and 7 illustrate plots of the log RNA size (in bases) versus Migration and demonstrate good linearity of response of each ladder. Base numbers were used to plot the calibration curve instead of MW since the MW information was not provided from the vendor of the ladders. The coefficient of determination (R^2) of RNA6000 ladder is 0.993273 while the R^2 of Promega RNA ladder is 0.988811, which is slightly less than 0.99. One of the possible reasons for the relatively low R^2 value of the calibration curves is the different composition of the nucleic bases in the sequence of each RNA marker, which leads to the deviations of the relationship of MW and base numbers of the RNA markers.

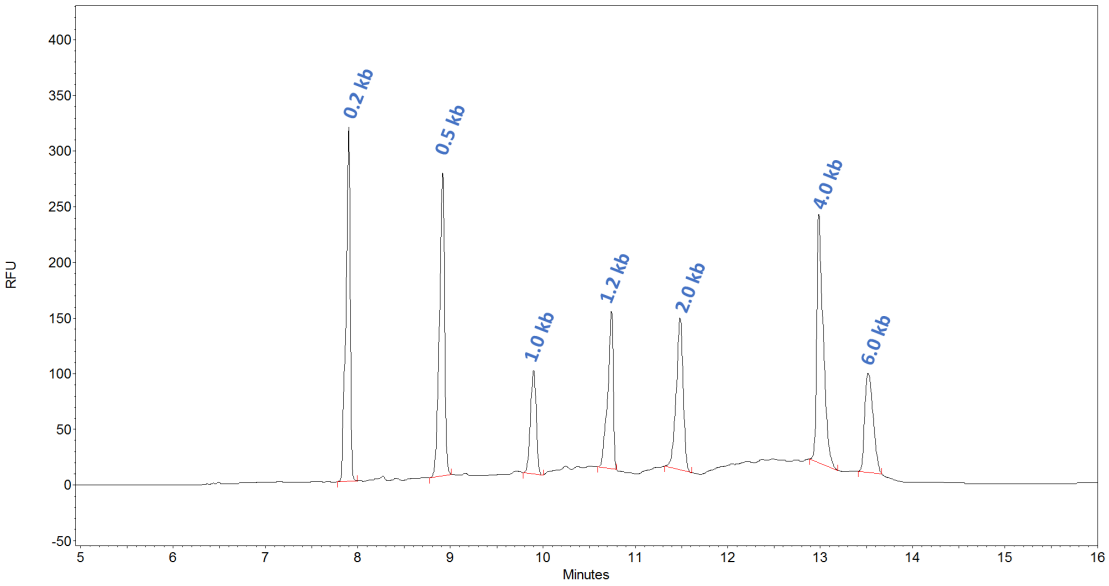

Figure 4. E-gram of the Thermo RNA 6000 Ladder with spiked in 1.2kb marker separated by the optimized method.

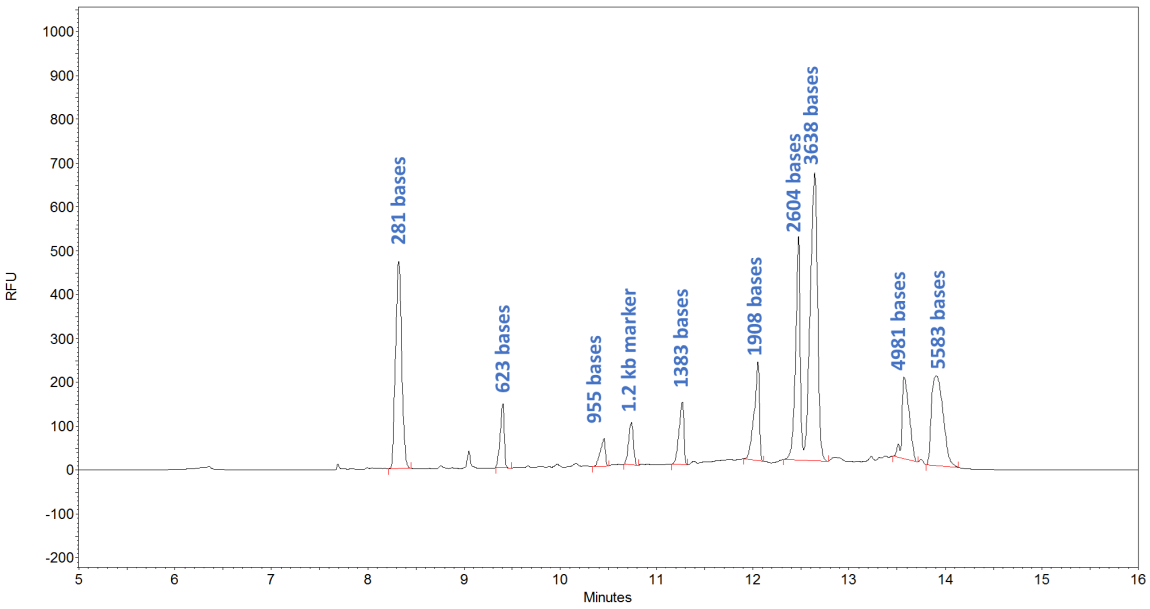

Figure 5. E-gram of the Promega RNA ladder spiked in 1.2 kb marker separated by the optimized method.

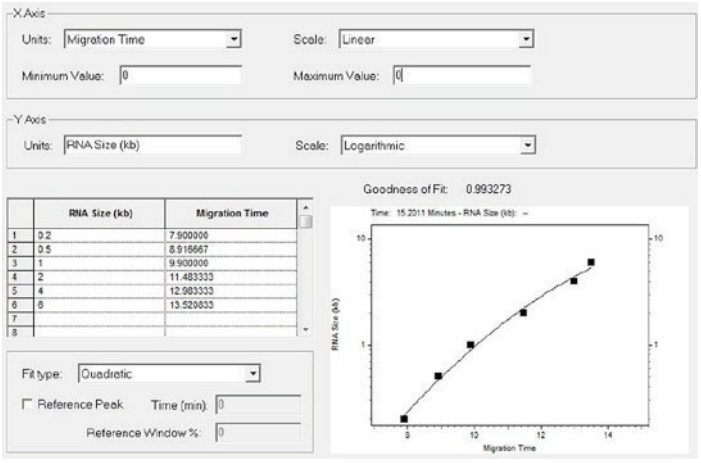

Figure 6. Calibration curve of log RNA Size in kb vs migration time of the Thermo RNA 6000 Ladder. R2 is 0.993273.

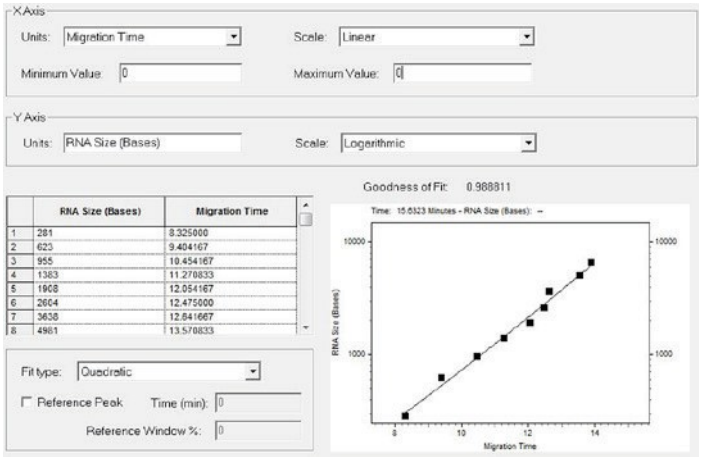

Figure 7. Calibration curve of log RNA Size in bases vs migration time of the Promega RNA ladder. R2 is 0.988811.

Repeatability

Both RNA ladder samples were injected eight times to demonstrate the repeatability of the method. Figure 1a and 1b show the overlay of the eight consecutive analyses of the Promega RNA ladder with spiked in 1.2 kb marker and the Thermo RNA6000 Ladder with spiked in1.2 kb marker, respectively. Table 1 summarizes the results of eight consecutive analyses of the Promega RNA ladder spiked in 1.2 kb marker. The relative standard deviation (%RSD) of the mobility for each RNA markers in the Promega RNA sample was < 0.1%, while the %RSD of the quantitative determination of the % corrected area of each marker was < 1.5%. Table 2 summarizes the results of repetitive injections of the Thermo RNA6000 ladder with spiked 1.2 kb marker. The relative standard deviation (%RSD) of the mobility of each RNA marker in the Thermo RNA6000 ladder sample was < 0.15%, while the %RSD of the % corrected area of each marker in the Thermo RNA6000 ladder sample was < 5%. The results demonstrate the excellent repeatability of this method.

	RSD%	
	CPA%	MT
281 bases	0.82	0.08
623 bases	1.26	0.09
955 bases	1.22	0.07
1200 bases	1.15	0.09
1383 bases	1.25	0.06
1908 bases	1.37	0.07
2604 bases	0.95	0.09
3638 bases	0.59	0.06
4981 bases	1.16	0.04
6583 bases	0.91	0.04

Table 1. The %RSD of the Migration Time (MT) and Corrected Peak Area Percentage (CPA%) of the 9 markers and a spiked in 1.2kb RNA molecule.

	RSD%	
	CPA%	MT
0.2 kb	2.13	0.15
0.5 kb	1.10	0.09
1.0 kb	1.46	0.07
1.2 kb	2.91	0.05
2.0 kb	3.01	0.11
4.0 kb	2.58	0.09
6.0 kb	4.68	0.09

Table 2. The %RSD of the Migration Time (MT) and Corrected Peak Area Percentage (CPA%) of the 6 markers and a spiked in 1.2kb RNA molecule.

Linearity, LOD and LOQ using LIF Detector

The 1.2 kb Positive Control RNA molecule with a known concentration of 0.5mg/mL was used for linearity, LOD and LOQ studies.

Figure 8 demonstrates the excellent linearity of detection response using the LIF detector, plotting peak area versus concentration (ng/mL) over the range of 0.33 ng/mL to 333 ng/mL for the 1.2 kb RNA Marker. The R2 is 0.9996. The LOQ of 1.2 kb RNA marker is 0.33 ng/mL (S/N=18), while the LOD of 1.2 kb RNA marker is 0.081ng/mL (S/N=4). The detector has approximately 3 orders of dynamic range.

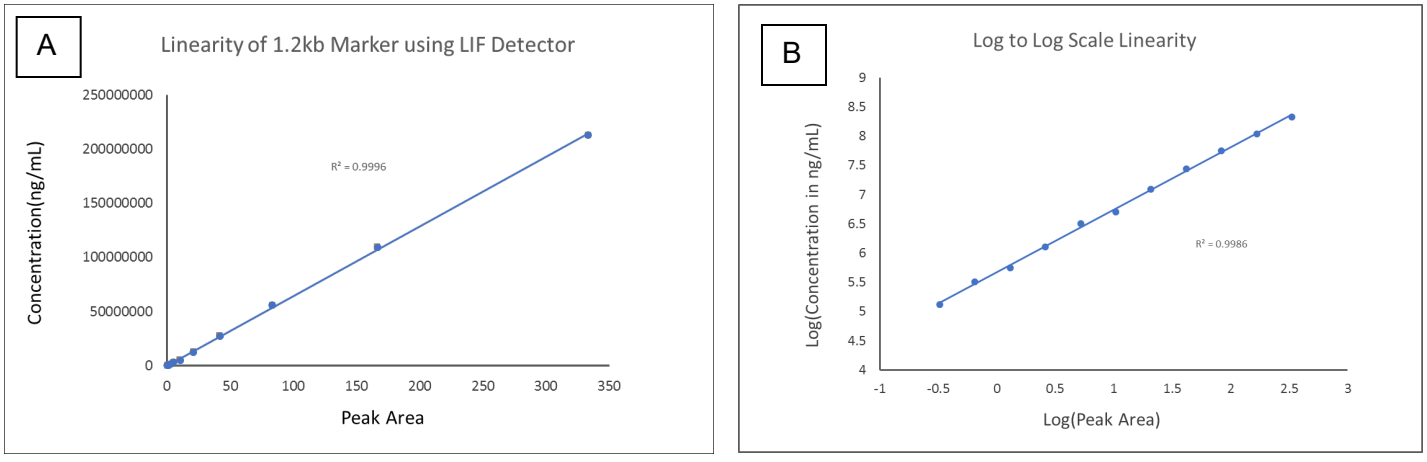

Figure 8. a) Linearity of detection response using LIF detector, plotting peak area versus concentration over the range of 0.33 ng/mL to 333 ng/mL of the 1.2 kb RNA Marker b) log (detection response) to log (concentration in ng/mL) plot to have the lower concentration part of the linearity plot visible.

Conclusions

In conclusion, a CGE-LIF method was optimized to resolve RNA from 0.2 kb to 6.5 kb in size. The optimized method demonstrates good resolving power, MT and CPA% repeatability, excellent linearity from 0.33 ng/mL to 333 ng/mL for 1.2 kb RNA Marker, LOQ as low as 0.33 ng/mL and provides the flexibility of further method modification.

Reference

1. Julia Khandurina, Hur-Song Chang, Bart Wanders, and Andras Guttman, *AN ULTRAFAST METHOD TO EVALUATE RNA QUALITY*

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Publication number: RUO-MKT-02-8017-B 08/2018

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