Comprehensive method development for a wide size range of RNA

Featuring the PA 800 Plus system and RNA 9000 Purity & Integrity Kit

Mervin Gutierrez, Fang Wang, Quincy Mehta, and Marcia Santos

SCIEX, USA

Introduction

The integrity and quality of the encapsulated nucleic acids used in cell and gene therapy are important to ensure the efficacy and safety of the therapeutic product. Current methods used to characterize the integrity and quality of these nucleic acids are complex and yield suboptimal analytical resolution and poor transferability. This work introduces a new chemistry and methods optimization strategy that is applicable to single-stranded RNAs with lengths ranging from 50 to 9000 bases, enabling scientists to achieve high-resolution and high-quality data (Figure 1). This technical note showcases the reproducibility for electrokinetic injection, a %RSD for migration time below 0.25% and %RSD for corrected peak area% less than 3 for fragments between 300 and 2000 bases for the single-stranded RNA (ssRNA) ladder.

Compared to traditional slab gel-based electrophoresis methods, capillary gel electrophoresis (CGE), combined with laser-induced fluorescence (LIF), offers superior resolution, shorter analysis time, automated operation and exceptional sensitivity. Compared to chip-based CE systems, we demonstrate easy method modification and optimization flexibility, allowing scientists to choose between project-specific methods to achieve optimal results and platform methods for higher throughput.

Capillary Temperature (°C)	Expected Capillary Run Life	Migration Time of 9 kb Peak (min)	Resolution between 7 and 9 kb peaks	EP Theoretical Plates (x 10^6)			
				Peak			
				1 kb	5 kb	7 kb	9 kb
25	130-150	21.18	3.62	1.19	1.37	1.67	1.11
30	100-120	19.78	4.31	1.22	1.98	1.87	1.12
35	70-80	18.51	5	1.24	2.02	1.92	1.39
40	50-60	17.35	5.1	1.22	1.95	1.78	1.39

Figure 1. Summary of assay performance criteria. Capillary run life, migration time for the 9 kb marker as a proxy for total assay time, resolution between 7 kb and 9 kb markers and theoretical plates were evaluated. N = 3 capillaries were evaluated at each temperature.

The SCIEX solution for extended range RNA purity and integrity analysis on the PA 800 Plus system.

This study evaluated an RNA ladder that ranged from 50 to 9000 bases, using the new RNA 9000 Purity & Integrity Kit and the PA 800 Plus system. The effects of varying conditions, such as injection mode and separation temperature, are described.

Key features

- Ready-to-use kit and method for single-stranded nucleic acid analysis
- Easy-to-optimize method strategy for samples containing small or large ssRNA fragments
- Ability to use either hydrodynamic or electrokinetic injections, depending on sample composition, and achieve great resolution
- Ability to separate a wide range of RNAs in a single separation
- Reproducible and reliable results from hydrodynamic injections, with migration time %CV <0.15% and corrected peak area% <4.5%
- Size estimation of RNA samples with easy to use and flexible modeling methods.

Methods

Materials: The RNA 9000 Purity & Integrity Kit (PN: C48231), containing the nucleic acid extended range gel, SYBR™ Green II RNA Gel Stain,* acid wash (regenerating solution), CE-grade water, the RNA ladder (50-9,000 bases) and LIF calibration solution, was obtained from SCIEX (Framingham, MA). The Pre-Assembled BFS Capillary Cartridge (30.2 cm bare-fused silica capillary, PN: A55625), SCIEX universal vials (PN: A62251), universal vial caps (PN: A62250) and PCR vials (PN: 144709) were also acquired from SCIEX. The universal vials, universal vial caps and PCR vials were used for sample and reagent loading. The CleanCap Cas9 mRNA (5moU, 4.5 kb) (PN: L-7206) was obtained from TriLink Biotechnologies (San Diego, CA). sgRNA (100 base) was obtained from Integrated DNA Technologies (IDT; Coralville, IA)

Sample preparation: The RNA 9000 molecular ladder was diluted with SLS, as described in the user manual. Specifically, 4 µL of ssRNA ladder was mixed with 96 µL of SLS. The mixture was then heated at 70°C for 5 minutes using a thermal cycler and immediately cooled on ice for at least 10 minutes. An 80 µL aliquot of the sample was transferred into the sample vial before being subjected to sample analysis. The Cas9 mRNA was diluted to 0.4 ng/µL and the sgRNA was diluted to 25 ng/µL with a 50% SLS in water. The diluted samples were denatured with heat using a thermal cycler at 70°C for 5 minutes, then immediately placed on ice until the time of injection for CE analysis using the same method as the ladders.

Instrument and software: The PA 800 Plus system (SCIEX), equipped with a laser-induced fluorescence (LIF) detector with a 488 nm solid-state laser and a 520 nm emission filter, was used for all separations. The LIF detector was calibrated according to the user guide. Data acquisition was performed using 32 Karat software version 10. Data files were exported in the ASCII format and later imported and processed using the BioPhase software to perform integration and calculations of signal intensity, corrected peak area and corrected peak area%.

Instrument set-up: The separation gel was prepared by mixing 10 µL of SYBR™ Green II RNA Gel Stain* with 5 mL of nucleic acid extended range gel. This separation gel was used for 8 injections, but this preparation can be scaled to the number of samples to be analyzed. The separation gel was prepared at the time of the experiment and leftover gel was discarded. The PA 800 Plus universal vials were then filled with 1.5 mL of separation gel and all other reagents needed for this application. The vials were properly capped before they were loaded onto the instrument, according to the reagent plate map provided in the user guide.[1] The separation method can be downloaded from the SCIEX website or created following the user guide.[1] For separation temperature screening, the capillary cartridge temperature was set to various temperatures between 25°C and 55°C for the pressure injection methods.

Results and Discussion

Effect of separation temperature

Separation temperature is an important consideration for CE method development. Higher temperatures can speed up the run time and affect peak resolution and shape. The PA 800 Plus system provides an accurate temperature control system that allows the user to set, evaluate and control the separation temperature to achieve an optimal balance of assay throughput and separation efficiency. Here, we assessed the separation of the ssRNA ladder with a size range of 50 to 9000 bases as a function of temperature. The overlaid electropherograms shown in Figure 2 show that separation time is reduced as the separation temperature increases. This result was expected, as an increase in temperature reduces the viscosity of the sieving matrix of the separation gel. Notably, the separation temperature also affected the resolution of the ssRNA markers in a manner dependent on the ssRNA fragment size. Higher temperatures improved the resolution of the larger RNA markers, whereas lower temperatures improved the resolution of the smaller RNA markers (Figure 1). Figure 3 shows the scatter plot of migration time, theoretical plates and peak widths for each of the ssRNA markers as a function of the separation temperature.

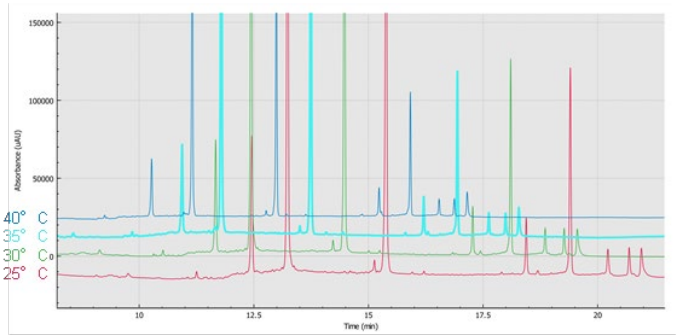

Figure 2. Overlaid ssRNA ladder electropherograms to compare separation at different temperatures. As the temperature increases, the migration time decreases and peak resolution is affected for all markers. Further details and quantitative comparison are presented in Figure 3. The capillary cartridge temperatures tested included 25°C, 30°C, 35°C and 40°C, from the bottommost to topmost electropherogram.

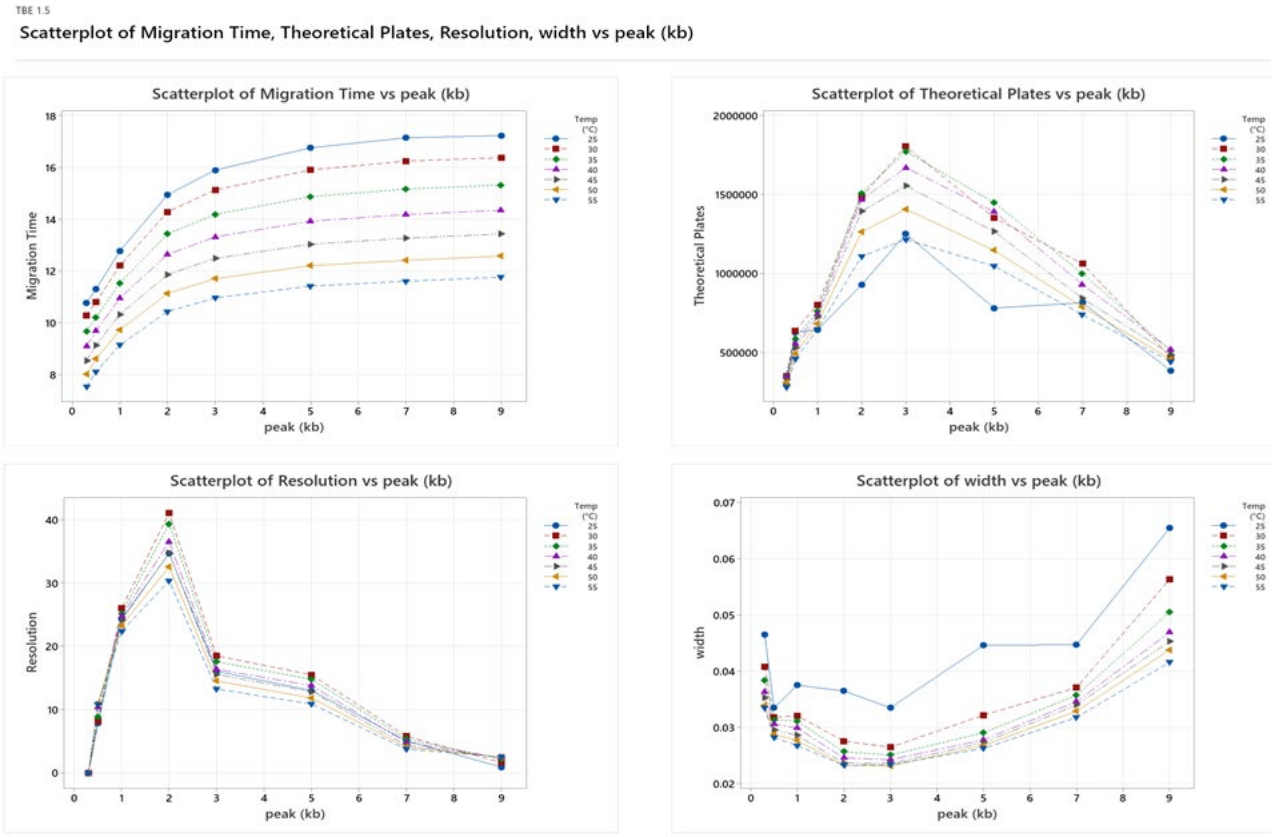

Figure 3. Quantitative analysis of ssRNA marker separation performance. Scatter plots of theoretical plates, peak resolution, peak width and migration time for all RNA markers at 7 different temperatures. Temperatures tested included 25°C (blue circle), 30°C (red square), 35°C (green diamond), 40°C (pink upward-facing triangle), 45°C (purple right-facing triangle), 50°C (yellow left-facing triangle) and 55°C (blue downward-facing triangle).

Interestingly, we found that increased separation temperature might detrimentally impact separation performance over time. N=3 cartridges were evaluated at temperatures between 25°C and 40°C to establish the optimal operation limit to preserve capillary longevity. Analysis revealed that 30°C is the best separation temperature to both maximize the run life of the capillary and resolve fragments that are in a size range of potential therapeutic relevance, between 1000 and 9000 bases.

Evaluation of injection modes

Either hydrodynamic (HDI) or electrokinetic (EKI) injections can be performed with the RNA 9000 Purity & Integrity Kit. For the HDI mode, pressure is used to drive a small volume of sample into the capillary, whereas for the EKI mode, an electric field is used to drive only the charged species of the sample into the capillary. EKI mode uses reverse polarity in CGE, in which residual electro-osmotic (EOF) flow moves away for the detector. As a result, only anionic species are introduced into the capillary. The different means by which sample is introduced to the capillary between these 2 methods results in HDI being representative of all sample components and EKI being biased towards species in the sample with higher mobility.[4] Figures 4 and 5 showcase the typical profile of ssRNA ladder using HDI and EKI modes, respectively.

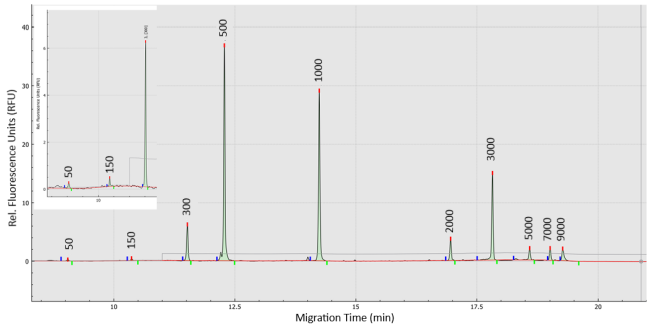

Figure 4. Separation results from the HDI method. Electropherogram of ssRNA ladder analyzed using HDI mode at 1psi for 5 seconds. The inset shows the y-axis zoom-in for markers smaller than 300 bases. Testing was performed on a 20/30 cm, 50 µm ID BFS capillary at 30°C capillary temperature and 200 V/cm field strength.

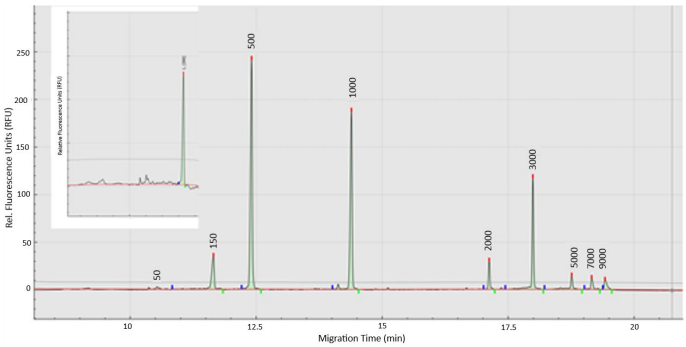

Figure 5. Separation results from the EKI method. The electropherogram of ssRNA ladder analyzed with EKI at 5 kV for 3 seconds. The inset shows the y-axis zoom-in for markers smaller than 300 bases. Testing was performed on a 20/30cm 50 µm ID BFS capillary at 30°C capillary temperature and 200 V/cm field strength.

The results indicated that both HDI and EKI modes yield similar profiles for markers larger than 300 bases. We observed increased peak widths of fragments between 50 and 300 bases using the EKI mode, but not HDI mode. The increase in peak widths results in decreased theoretical plates and might have a negative impact on the ability to assess purity fragments with sizes between 50 and 150 bases. Therefore, a pressure injection should be considered if the analyte of interest is smaller than 300 bases. Conversely, EKI is inherently a stacking technique because it induces a pre-concentration of the sample band during the injection, resulting in this case, in a 5-fold increase in response signal. Furthermore, EKI mode is recommended when working with samples present at low concentrations and containing RNA fragments above 300 bases.

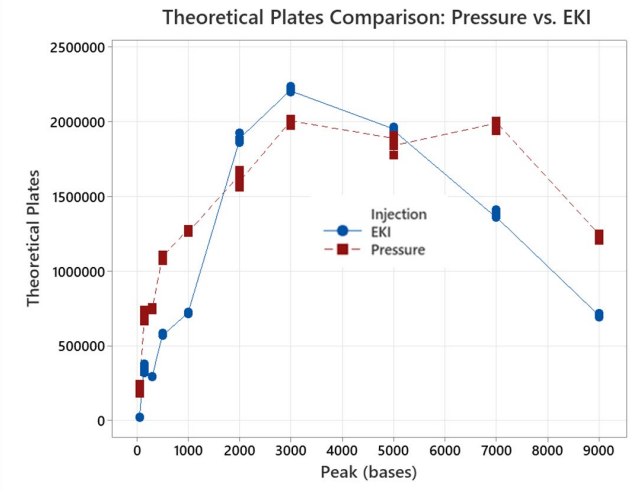

Figure 6. Comparison of theoretical plates for each ssRNA marker using EKI and HDI. The EKI method (blue circle) was implemented at 5 kV for 3 seconds and the HDI method (red square) was implemented at 1psi for 5 seconds. Testing was performed on a 20/30cm 50 µm ID BFS capillary at 30°C capillary temperature and 200 V/cm field strength.

It is possible to impart stacking capability to the HDI mode by injecting a small water plug before the injection of the sample. This technique allows for a significant improvement in peak shape and resolution for the ssRNA fragments smaller than 300 bases. A quantitative evaluation of the theoretical plates between EKI and HDI modes is shown in Figure 6. Based on these factors, HDI is recommended for analytes smaller than 500 bases.

Assay repeatability

The repeatability of the method was evaluated at 30°C separation temperature for the 2 injection modes. Sixteen injections were performed for each prepared sample, using EKI (Figure 7) or HDI (Figure 8). The average and %RSD for migration time and corrected peak area were calculated for each fragment size marker peak. Only fragments larger than 300 bases were considered in the calculations for the EKI mode (Table 1), whereas all 10 fragment markers were evaluated for the HDI mode (Table 2).

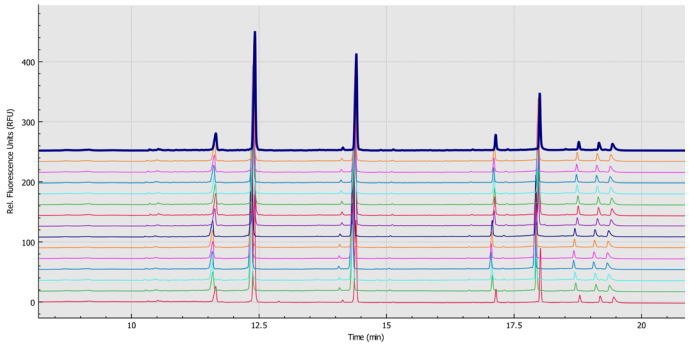

Figure 7. Assay repeatability using the EKI method. Overlaid electropherograms of 16 ssRNA ladders analyzed with the EKI method at 5 kV for 3 seconds. Testing was performed on a 20/30cm 50 µm ID BFS capillary at 30°C capillary temperature and 200 V/cm field strength.

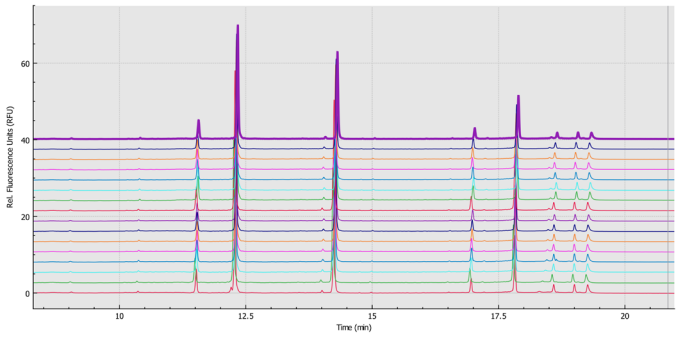

Figure 8. Assay repeatability using the HDI method. Overlaid electropherograms of 16 ssRNA ladders analyzed with the HDI method at 1 psi for 5 seconds. Testing was performed on a 20/30cm 50 µm ID BFS capillary at 30°C capillary temperature and 200 V/cm field strength.

Table 1. Repeatability evaluation of the EKI mode for separation of ssRNA ladders.

Peak ID	300	500	1000	2000	3000	5000	7000	9000
Average for MT	*11.62*	*12.38*	*14.37*	*17.10*	*17.96*	*18.72*	*19.12*	*19.39*
%RSD for MT	*0.23*	*0.20*	*0.18*	*0.18*	*0.19*	*0.19*	*0.19*	*0.19*
Average for corrected peak area%	*8.96*	*40.46*	*29.15*	*3.04*	*11.93*	*2.62*	*1.91*	*1.93*
%RSD for corrected peak area%	*7.20*	*1.54*	*2.30*	*7.46*	*4.00*	*9.62*	*10.99*	*6.42*

Table 2. Repeatability evaluation of the HDI mode for separation of ssRNA ladders.

Peak ID	50	150	300	500	1000	2000	3000	5000	7000	9000
Average for MT	*9.03*	*10.38*	*11.54*	*12.31*	*14.27*	*16.98*	*17.84*	*18.61*	*19.02*	*19.28*
%RSD for MT	*0.09*	*0.15*	*0.15*	*0.15*	*0.15*	*0.13*	*0.13*	*0.13*	*0.13*	*0.13*
Average for corrected peak area%	*0.48*	*0.54*	*7.77*	*40.59*	*30.07*	*3.10*	*11.70*	*2.15*	*1.51*	*2.09*
%RSD for corrected peak area%	*10.56*	*4.82*	*1.86*	*1.43*	*1.74*	*1.44*	*1.24*	*4.24*	*2.00*	*2.71*

Table 3. Size estimation of two RNA samples using different modeling methods

		Point-to-point		Log (y) 50-500		Log (y) 500-3000		Log (y) 3000-9000	
Peak ID	Theoretical length (b)	Calculated length (b)	% Difference	Calculated length (b)	% Difference	Calculated length (b)	% Difference	Calculated length (b)	% Difference
sgRNA	100	118	18.0	99	1.0	244	144.0	8	92.0
Cas9 mRNA	4500	5196	15.5	40285	795.2	3509	22.0	4931	9.6

When EKI mode was used, the migration time of the fragment markers increased slightly (Figure 7), resulting in a minimum impact to the %RSD for migration time <0.25%. Due to the small contribution of the markers at 50 and 150 bases to the total corrected peak area, the reported corrected peak area% for markers above 300 bases is not affected when the different injection modes are used. The separations using EKI mode show a higher %RSD for the corrected peak area% for each marker, possibly due to the inherent bias introduced by EKI.[4] The average total corrected peak area for the pressure injection was 4.52, compared to 34.99 for the EKI injection, which is consistent with the increased sample load typical of an EKI injection.

Size Estimation of RNAs

The BioPhase Data Analysis module provides 6 different fitting types for marker fittings. Similar to molecular weight estimation by CE, RNA size estimation also uses external size markers running in the same sequence rather than an internal marker.

Among the 6 fittings, the point-to-point fitting is used in size estimation for RNA samples across the entire size range of the 10 markers. As described earlier, the gel buffer provides the best resolution for RNAs in the size range of 500 to 3000 bases, with slightly decreased resolution in the size range of 50-500 and 3000 -9000 bases. Using the point-to-point size estimation method assumes a linear correlation between peak migration time and the size within each pair of markers. This method allows a size estimation of different-sized peaks in one run. Additionally, this is the model that has been used and demonstrated to fit the purpose of having a calculated size that corresponds well to the theoretical value without additional experimental procedures. The accurate size determination and sequence confirmation of an mRNA still relies on other orthogonal technology.

Alternatively, three different slopes are observed for Log(y) fittings plot in the size range of RNA ladder separation: 1) 50 to 500 bases, 2) 500 to 3000 bases, and 3) 3000 to 9000 bases. When the three different size ranges are fitted separately, a log (y) fitting can also be used for size estimation. (Figure 9). The size estimation model should be chosen during the method development process. Figure 10 shows the setup for size estimation using external markers. The point-to-point size estimation uses all 10 markers (Figure 10 A) and the peak to be

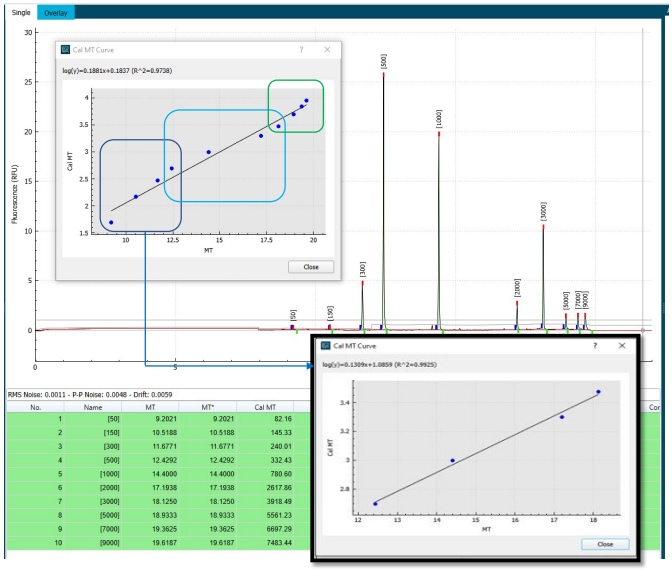

Figure 9. Curve fitting of RNA markers using Log (y) model. Three different slopes were observed when all 10 markers are included in one fitting. The three different slopes are indicated with three different colored rectangles. 1) 50 to 500 bases (dark blue), 2) 500 to 3000 bases (light blue), and 3) 3000 to 9000 bases (green). The adjusted log (y) fitting for the range of 50-500 bases only is shown in the bottom insert figure.

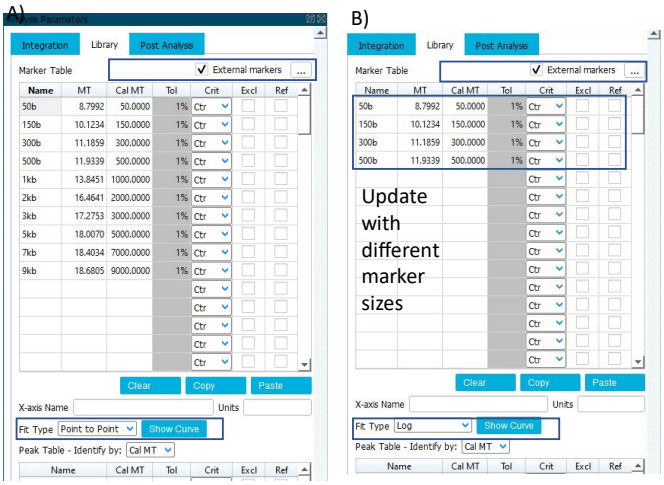

Figure 10. External size marker calibration set up. A). Example for setting up point-to-point size estimation modeling using ssRNA ladder in the kit. B) Example for setting up log(y) size estimation modeling using markers in size range of 50-500 bases. If other markers are needed, different migration time and CalMT (size) can be updated. In the results shown in Table 3, all three different slopes 1) 50 to 500 bases (dark blue), 2) 500 to 3000 bases (light blue), and 3) 3000 to 9000 bases (green) were used.

calculated must have a migration time in the range of size markers, there's no projection calculation outside of the marker ranges. The log (y) size modeling can only be used with the three different size ranges listed above. Figure 10 B shows an example of using the ladder 50-500 bases for the size estimation, however, using log fittings allows projection calculation outside of the marker size limit. However, the accuracy of these projections is not good (Table 3). In general, the point-to-point fitting provides an easy and good estimation of RNA size.

Conclusions

- The RNA 9000 Purity & Integrity Kit provides high-resolution separation for RNAs ranging from 50 to 9000 bases
- The method can be easily optimized and tailored to different RNA sizes, assay requirements, and sample matrices by adjusting separation temperature and injection mode
- The assay showed high repeatability and consistency under the conditions evaluated
- The data analysis software allows size estimation of RNA samples using the kit size markers as external calibration

References

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