Application Note

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Single-Guide RNA Quality Assessment with the Agilent 5200 Fragment Analyzer System

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Abstract

One method of CRISPR/Cas9 gene editing is the delivery of Cas9 complexed *in vitro* with a single-guide RNA (sgRNA). Traditionally, quality control analysis of sgRNA prior to Cas9 complexing is performed by agarose gel electrophoresis. The Agilent 5200 Fragment Analyzer system facilitates easy visual quality assessment of sgRNA integrity with excellent separation of the linear and secondary structure fragments on both the Agilent HS RNA kit (15 nt) and the Agilent Small RNA kit. Reliable capillary electrophoresis with the 5200 Fragment Analyzer system provides consistent quantification and sizing for each sgRNA sample.

Introduction

Efficient CRISPR/Cas9 gene editing can be accomplished by direct delivery of complexed Cas9 and single-guide RNA (sgRNA) to a cell line, organism, or tissue. This process relies on *in vitro* transcription of the sgRNAs from engineered DNA constructs such as gBlocks. Synthesis of guide RNAs using this process usually produces enough material for many transfections. However, multiple freeze thaws, improper handling, or contamination can degrade sgRNA preventing efficient gene editing.

The quality of sgRNA prior to Cas9 complexing and transfection is traditionally analyzed by agarose gel electrophoresis. While this process can determine whether an sgRNA is extensively degraded, the resolution and sensitivity of agarose gels does not allow for detection of low levels of degradation that can affect editing efficiency. Additionally, RNA agarose gels require the use of hazardous materials such as formaldehyde and DEPC. The Agilent 5200 Fragment Analyzer system automates sgRNA quality control steps without the use of hazardous denaturants while providing enhanced resolution that enables the detection of the smallest amounts of degradation. We compared detection of sgRNA degradation and sizing between agarose gel electrophoresis and two kits for the 5200 Fragment Analyzer system; the Agilent HS RNA kit (15 nt) and the Agilent Small RNA kit.

Experimental

The experiments in this study were done using an Agilent 5200 Fragment Analyzer system and can be replicated with comparable results on Agilent 5300 and 5400 Fragment Analyzer systems.

In vitro transcription of sgRNA

Seven sgRNAs (Table 1) were transcribed from double stranded DNA (dsDNA) gBlock gene fragments (Integrated DNA Technologies) using the T7 high-yield RNA synthesis kit (New England BioLabs HiScribe, #E2040S). The 30 µL reaction mixture of 10 µL NTP buffer mix, 2 µL T7 RNA polymerase mix, 1 pmole gBlock, and nuclease-free water was incubated at 37 °C for 16 hours. To remove the gBlock DNA, 20 µL of nuclease-free water and 2 µL NEB DNase I was added to the *in vitro* transcription reaction and incubated at 37 °C for 15 minutes. Following DNase treatment, sgRNAs were purified using RNA clean and concentrator (Zymo Research, #R1017) and quantified using the Qubit RNA HS assay (Thermo Fisher Scientific, #Q32852).

Heat denaturing and analysis

The sgRNA samples were heat denatured at 70 °C for 10 minutes. The heat denatured sgRNA samples were diluted to 1 ng/µL and analyzed on the Agilent 5200 Fragment Analyzer system with the Agilent HS RNA kit (15 nt) (p/n DNF- 472) and the Agilent Small RNA kit (p/n DNF-470). Agarose gel electrophoresis analysis was performed using the rapid formaldehyde-free RNA gel kit (Amresco, #1B1384-KIT) by diluting 2.5 µL of 288 ng/µL sgRNA into 5 µL Amresco formaldehyde-free RNA gel loading buffer 2X and 2.5 µL nuclease-free water. The entire 10 µL sample was loaded on the agarose gel and separated at 80 V for about 40 minutes using the Agilent RNA Ladder (p/n DNF-386). Agarose gels were visualized using a UV transilluminator. Qubit 2.0 was used for quantification of all samples.

Heat degradation and analysis

Undiluted sgRNA (about 300 ng/µL) was degraded at 90 °C for 0, 15, 30, 60, 90, and 120 minutes. At each time point, 1 µL of sample was diluted to a concentration of 1 ng/µL and analyzed on the 5200 Fragment Analyzer system with the HS RNA kit and the Small RNA kit. Additionally, agarose gel electrophoresis was performed following the protocol utilized for heat denatured sgRNA.

Table 1. Percent G-C content of the guide sequence and linear peak size of the sgRNAs used in this study.

sgRNA No.	Guide sequence	% G-C in guide sequence	% G-C in total vector	Linear peak size (nt)		Expected peak size (nt)
				Small RNA kit	HS RNA kit	
1	ACTGGGCGGCAGCATAGTGA	60	43.4	82	133	100
2	GAGAAGAAGCCTATAAAATA	30	37.3	90	135	100
3	AGTGGGACTTTGGAAATACA	40	39.4	80	129	100
4	AAGGTGACCGTCCTGGCTTT	55	42.4	68	113	100
5	TGTCAAGAGTTGACACATTG	40	39.4	89	141	100
6	GTGGGACTTTGGAAATACAA	40	39.4	84	132	100
7	AATTAATTAACCACGAAGCG	35	38.4	90	147	100

Results and discussion

Heat denaturing sgRNA

The overall quality of an RNA preparation can be assessed by various forms of gel electrophoresis. Heat denaturing RNA before separation by electrophoresis reduces extensive secondary structures formed by intramolecular base pairing, which may inhibit size-dependent migration of RNA fragments.

Separation of sgRNAs with and without heat denaturation was performed on the 5200 Fragment Analyzer system with the HS RNA kit and Small RNA kit. sgRNAs are capable of folding into many different secondary structures. The presence of denaturing formamide in the diluent marker of the two kits was adequate to limit sgRNA secondary structure formation without heat denaturation in most samples. Without heat denaturation, the HS RNA kit separated sgRNA #7 into a major peak at 147 nt and minor peak at 543 nt (Figure 1A), while the Small RNA kit separated sgRNA #7 into a major peak at 90 nt

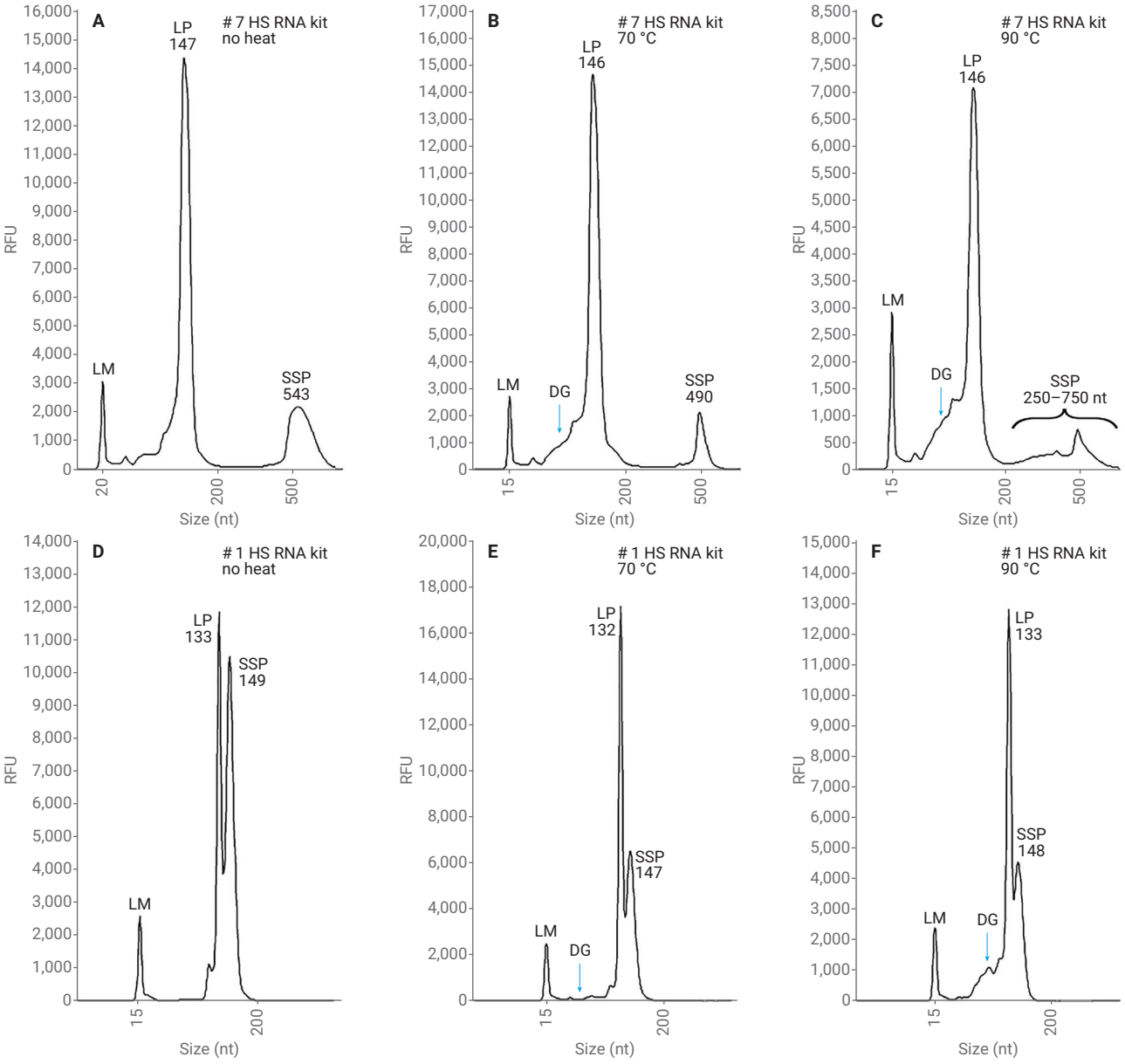

Figure 1. sgRNA samples separated with the Agilent HS RNA kit (15 nt) on the Agilent 5200 Fragment Analyzer system and treated without heat, heat denatured (70 °C for 10 minutes) or degraded (90 °C for 30 minutes). (A) sgRNA #7 without heat; (B) sgRNA #7 heat denatured; (C) sgRNA #7 degraded; (D) sgRNA #1 without heat; (E) sgRNA #1 heat denatured; (F) sgRNA #1 degraded. Linear peak (LP), secondary structure peak (SSP), degradation (DG), LM = lower marker.

and minor peaks from 300 to 500 nt (Figure 2A). sgRNAs #4 and #5 (example not shown) displayed a single peak with both kits, while sgRNAs #1, 2, 3, and 6 separated as a split peak with the HS RNA kit (sgRNA #1, Figure 1D) and two distinct peaks with the Small RNA kit (sgRNA #1, Figure 2D). Separation of the split peak most likely occurred because the Small RNA kit offers better resolution for small RNA. Heat denaturing slightly changed the separation profile of sgRNAs with two peaks (Figure 1B, 1E, 2B, and 2E) by decreasing the height of the second peak when compared to nonheat denaturing (Figure 1A and D; Figure 2B and D). Therefore, major peaks were labeled as linear peaks (LP), while minor peaks whose height and area decreased or disappeared due to heat denaturing were labeled as secondary structure peaks (SSP). Comparison of the sgRNA separation profiles with and without heat denaturation suggested that, sgRNA #7 exhibited a major linear structure peak and minor secondary structure peaks. The second split peak in sgRNA #1, 2, 3, and 6 was due to minor secondary structures.

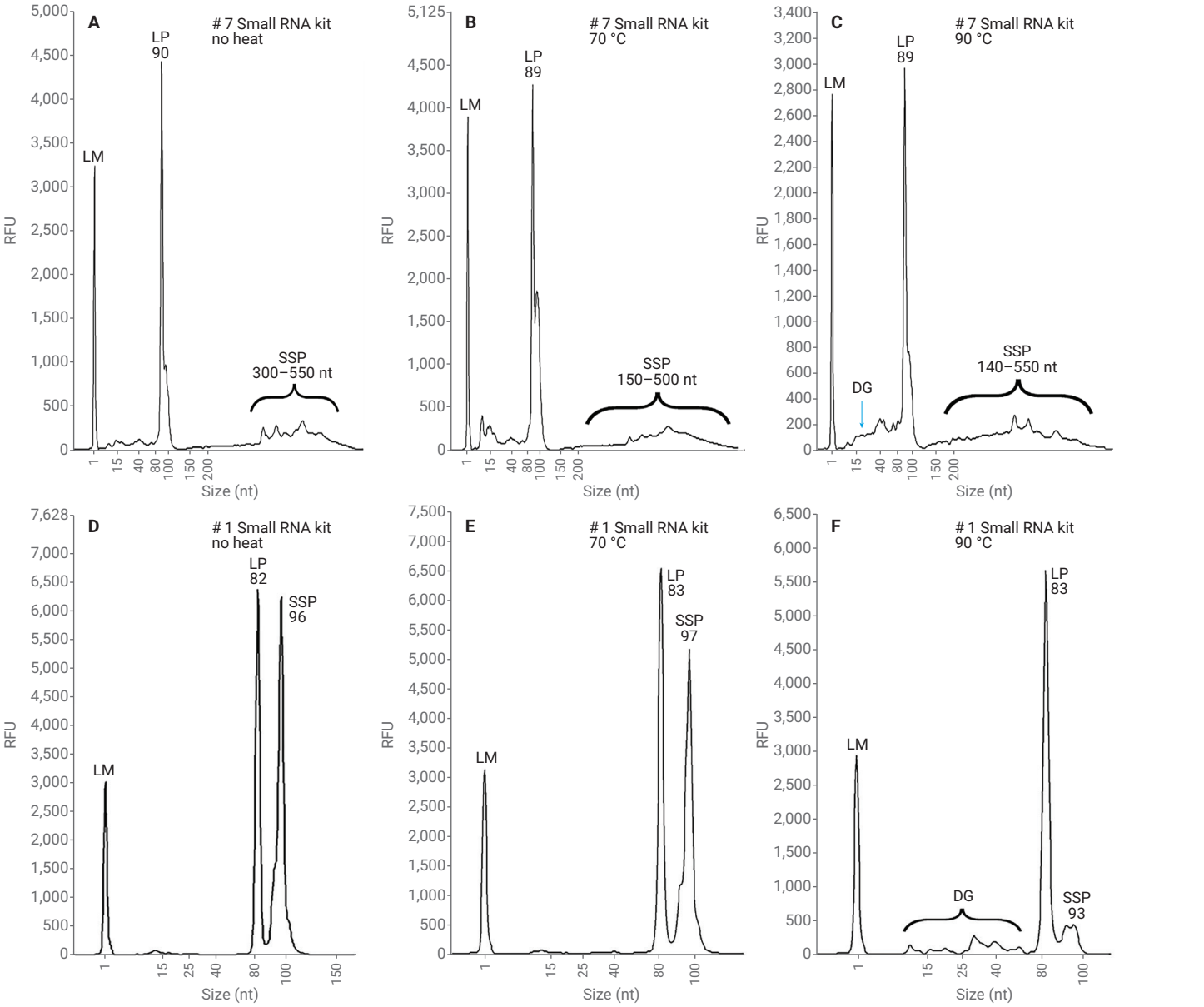

Figure 2. sgRNA samples separated with the Agilent Small RNA kit on the Agilent 5200 Fragment Analyzer system and treated without heat, heat denatured (70 °C for 10 minutes) or degraded (90 °C for 30 minutes). (A) sgRNA #7 without heat; (B) sgRNA #7 heat denatured; (C) sgRNA #7 degraded; (D) sgRNA #1 without heat; (E) sgRNA #1 heat denatured; (F) sgRNA #1 degraded. Linear peak (LP), secondary structure peak (SSP), degradation (DG), LM = lower marker.

After heat denaturation, sizing of the linear sgRNA peak for all samples remained consistent with both kits on the 5200 Fragment Analyzer system (Figure 1B, 1E, 2B, 2E). The only significant change in sizing was the secondary structure peak from sgRNA #7, which was reduced in size from 543 to 490 nt on the HS RNA kit (Figure 1B) and produced a wider smear on the Small RNA kit. Samples with secondary structure peaks (sgRNA #1, 2, 3, 6, 7) generally demonstrated an increase in percent concentrations of the linear peaks (Figures 4 and 5) and a decrease in percent concentration of the secondary structure peaks with both kits. However, sgRNAs with single peaks (sgRNA #4 and 5) tended to have a decrease in percent linear concentration and an increase in degradation (Figure 4 and 5). Degradation (DG), after heat denaturation was noted as an increased shouldering on the left side of the linear sgRNA peak, which increased from 0 to 3 % (Figure 1B, 1E, 2B, 2E).

Separating sgRNA #7 by agarose gel electrophoresis after heat denaturation, eliminated multiple secondary structure smears and separated two bands representing a linear and secondary structure (Figure 3A, lanes 4 and 5) which corresponded to similar sizes on the 5200 Fragment Analyzer system (Figure 1A). The two bands appeared to be of equal intensity on the agarose gel, unlike the two peaks separated on the 5200 Fragment Analyzer system. Agilent ProSize data analysis software enabled direct evaluation of the peak concentrations instead of an estimated concentration provided by the agarose gel. The 5200 Fragment Analyzer system was able to resolve the closely migrating linear and secondary structure peaks in sgRNA #1. The agarose gel would be unable to distinguish between the two bands due to a lack of resolution.

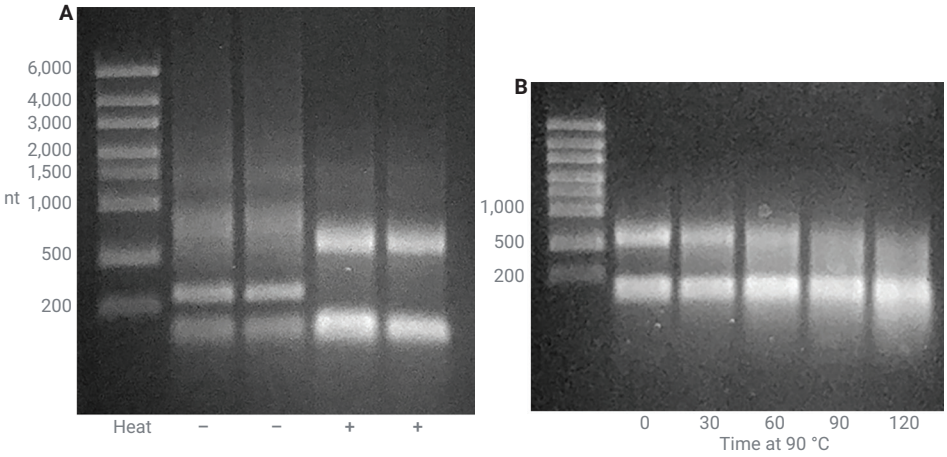

Figure 3. sgRNA #7 separated by agarose gel electrophoresis. (A) Lanes 2 and 3 received no treatment (–) and lanes 4 and 5 were heat denatured (+) at 70 °C for 10 minutes; (B) Degraded at 90 °C for 0, 30, 60, 90, 120 minutes.

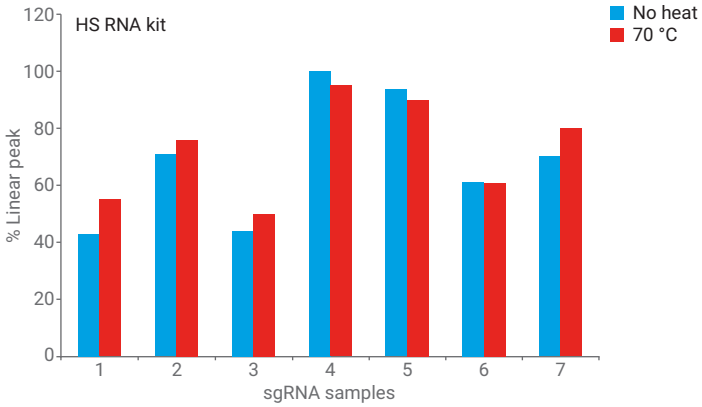

Figure 4. Percent concentration of the sgRNA linear peak with and without heat denaturation separated on the Agilent 5200 Fragment Analyzer system with the Agilent HS RNA kit (15 nt).

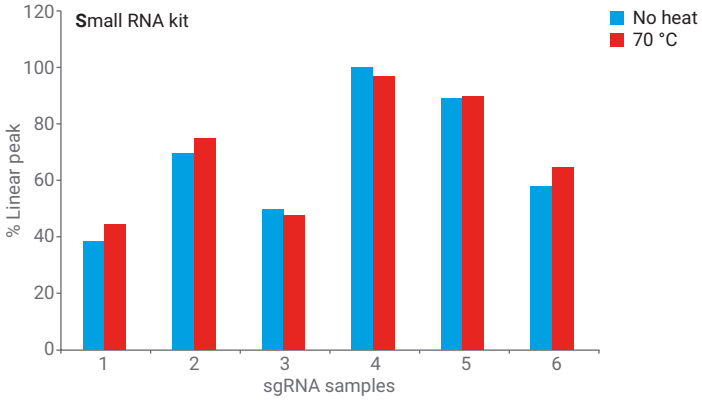

Figure 5. Percent concentration of the sgRNA linear peak with and without heat denaturation separated on the Agilent 5200 Fragment Analyzer system with the Agilent Small RNA kit.

Degradation

Quality control analysis of sgRNA is utilized to detect degradation that often occurs through contamination with RNase or repeated freeze thawing. The 5200 Fragment Analyzer system clearly detected degradation of the sgRNA after 30 minutes at 90 °C with both the HS RNA kit (Figure 1C, 1F) and Small RNA kit (Figure 2C, 2F). All samples displayed an increased shouldering on the left side of the linear peak. Degradation decreased the height (sgRNA #1; Figure 2C, 2F) or broadened the secondary structure peak (sgRNA #7; Figure 1C, 1F). In order to separate the secondary structure peak in sgRNA #7, the Small RNA kit required an extended run time (Figure 2C). The sgRNA degradation patterns are more clearly defined in the shouldering of the linear peak in the Small RNA kit compared to the HS RNA kit.

Separation of degraded sgRNA #7 by gel electrophoresis demonstrated two clearly separated bands (Figure 3B). Detection of degraded sgRNA after 30 minutes of heat degradation on the agarose gel was more difficult to visualize than on the 5200 Fragment Analyzer system. There appeared to be negligible amounts of degradation with only a slight smear between the two bands after 30 minutes. Low gel resolution only allowed for estimation of the peak size and degree of degradation, while the 5200 Fragment Analyzer system automatically reported a peak size with easy visualization and analysis of sgRNA degradation.

Sizing and concentration

The Small RNA kit reported a more accurate linear peak size than the HS RNA kit when compared to the expected sgRNA size of 100 nt (Table 1). This was attributed to the difference in ladder fragment sizes between the two kits. The Small RNA Ladder was designed for small-size RNAs ranging from 15 to 200 nt; whereas the HS RNA Ladder ranges from 200 to 6,000 nt. Since the linear sgRNA peak falls in the middle of the small RNA Ladder, the Small RNA kit is a more suitable choice for sgRNA sizing, however the run time may need to be extended to allow separation of secondary structures.

As G-C bonds are stronger than A-T bonds, G-C rich regions often report inconsistent sizing results due to secondary structure formation. The percent G-C content in the 20 nt guide sequence ranged from 30 to 60 %, but overall did not greatly alter the total percent G-C content (37 to 43 %) and did not have a direct correlation with the linear peak size of the sgRNAs (Table 1). Both the HS RNA kit and the Small RNA kit reported similar percent linear peak concentrations (Figure 4 & 5) and total concentrations across all samples with (data not shown) or without heat treatment (Figure 6). Either kit can be used to determine the concentration of sgRNAs. The agarose gel can only provide an estimated size for each band (Figure 3B). Further software analysis is required to obtain concentration estimates and more accurate results overall for agarose gel electrophoresis.

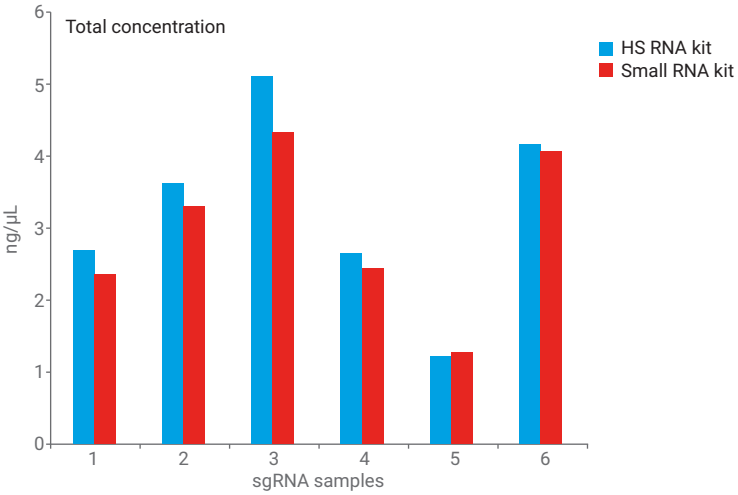

Figure 6. Comparison of total concentration between Agilent HS RNA kit (15 nt) and Agilent Small RNA kit with no heat treatment.

overall for agarose gel electrophoresis.

Conclusions

Table 2 summarizes sgRNA analysis by parallel capillary electrophoresis on the 5200 Fragment Analyzer system with the HS RNA kit (15 nt) and small RNA kit, and by agarose gel electrophoresis. The HS RNA kit provided efficient and excellent separation of sgRNA and easily detected sgRNA degradation. The Small RNA kit reported a more accurate nucleotide size for the linear sgRNA peak and easily detected degradation, though it required a longer run time to separate the 500 nt secondary structure peak. Both kits reported similar total concentrations and percent linear peak concentrations.

Table 2. Summary of sgRNA analysis methods.

	Run time	Average size of 100 nt linear peak	Comments
Small RNA kit 5 to 200 nt	35 minutes	86 nt	• Accurate nucleotide sizing • Easy visual detection of minimal degradation • Good separation of sgRNA peaks • Designed specifically for small RNA
HS RNA kit (15 nt) 200 to 6,000 nt	40 minutes	133 nt	• Visual detection of minimal degradation • Good separation of sgRNA peaks • Can be used to evaluate Total RNA
Agarose gel	45 minutes	Estimation only	• Cannot separate closely spaced bands • Minimal degradation not easily visualized • Requires heat denaturing of sgRNA

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