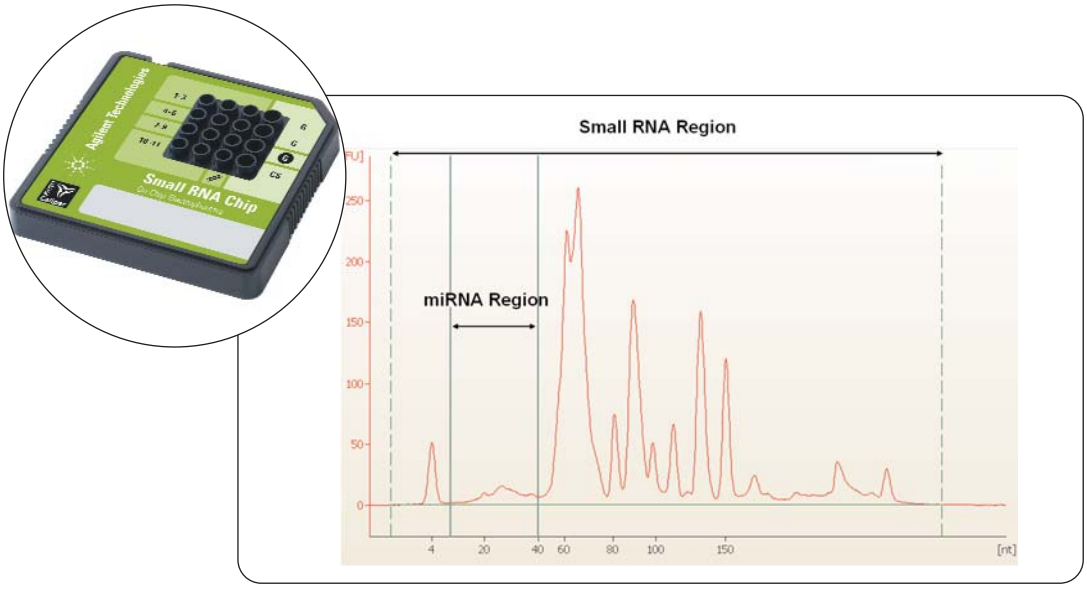
Performance of the Agilent Small RNA assay

Technical Note



Abstract

Small nucleic acids ranging in size from 6 to 150 nucleotides can be analyzed running the Small RNA assay on the Agilent 2100 bioanalyzer. This Technical Note describes the performance of the Small RNA assay. The Small RNA assay can:

- Visualize miRNA, Small RNA, oligo nucleotides from 6 - 150 nt for verifying sample integrity
- Quantify miRNA in the concentration range of 50 - 2000 pg/µL relative to an external standard, for verifying sample enrichment and purity
- Automate sample quantitation, sizing and purity determination

Agilent Technologies

Introduction

The role of small RNA molecules as key regulators of mRNA turnover and translation has been well established. Recent advances indicate that micro RNA (miRNA) and other small RNAs play an important role in cell proliferation, apoptosis and differentiation[1, 2]. As a unique class of small sized nucleic acid with very low abundance, small RNA requires special tools for accurate and sensitive analysis.

The new Agilent Small RNA assay provides a faster and much more sensitive alternative to agarose and polyacrylamide gels analysis for detecting small RNA. It can detect miRNA down to the 50 pg/µL level in the 6 - 150 nt size range. Sample types that can be analyzed are:

- total RNA
- purified small RNA (fraction < 200 nt)
- synthetic RNA oligonucleotides
- synthetic DNA oligonucleotides

The Small RNA assay is designed to run on the Agilent 2100 bioanalyzer.

Material and methods

RNA and ssRNA oligo samples

Mouse thymus, mouse liver, human placenta total RNAs, and transfer RNA (tRNA) were obtained from Ambion, Inc. Purified small RNA was isolated from human embryonal kidney (HEK) cells using the MirVana kit (Ambion, Inc), according to manufacturer's instructions. HEK cells were obtained from DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. The oligo mix was an equimolar mixture containing 80 different synthetic RNA oligonucleotides in the size range of 18-23 nucleotides selected from the Sanger Human miRBase Sequence Database (Wellcome Trust Sanger Institute). The 20-mer RNA oligo (3'AGCUGAGGUCAAGAUCUAAC 5') was obtained from Metabion International AG.

Chips and reagents

RNA samples were run on the commercially available Small RNA kit from Agilent, (Agilent part number 5067-1548), which contains all necessary chips, reagents and the protocol.

Data analysis

All Small RNA assay runs were carried out with the Agilent 2100 expert software, which offers automated data analysis for concentration and size determination.

Results and discussion

Sample types run on the Small RNA assay

Total RNA – visualizing small RNA in total RNA samples

To visualize the small RNA content of a total RNA extract, a mouse thymus total RNA sample with a concentration of 75 ng/uL was analyzed with the Small RNA assay (figure 1). The miRNA area between 6 and 40 nucleotides, is delineated by two vertical lines and the small RNA area by two dashed lines. The highest peak in the small RNA area with a size between 40 and 80 nt mostly represents transfer RNA. The larger peaks represent others small RNA species present in total RNA (pre-miRNA, 5S, 5.8S ribosomal RNA, etc). The small RNA content of a total RNA extract varies between different cell lines, and tissues types, and is highly dependent on

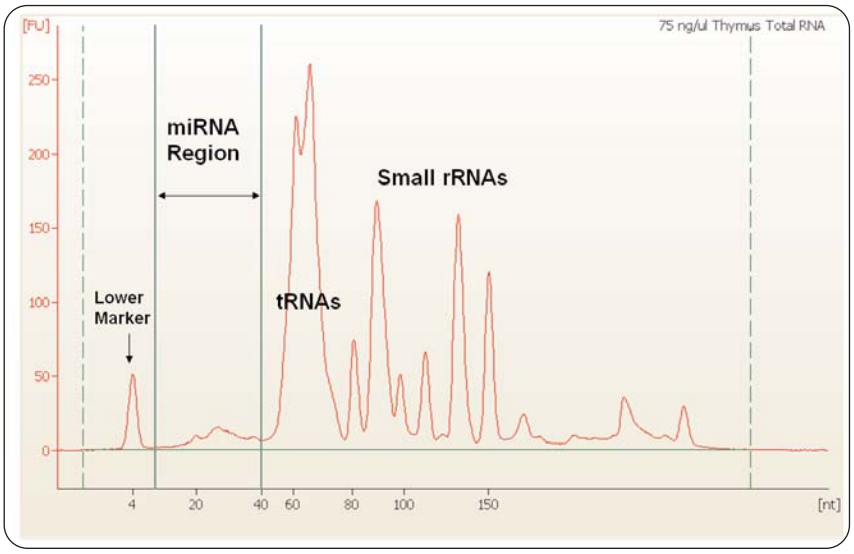


Figure 1
Electropherogram of total RNA sample, showing the small RNA content of 75 ng/µL Thymus total RNA (miRNA, tRNA, etc).

the way the total RNA has been purified. Since degradation of total RNA can produce fragments in the small RNA size range (up to 200 nt), it is suitable to cross check the total RNA sample integrity on the RNA 6000 Nano assay.

Small RNA – visualizing small RNA after purification from total RNA

Small RNA extracted from HEK cells were used to visualize purified small RNA samples (enriched below 200 nucleotides). Five ng of purified small RNA were analyzed with the Small RNA assay. The resulting electropherogram is shown in figure 2. The major content of the purified small RNA is mostly transfer RNA represented by the highest peak. The very low signal in the miRNA region, points out that the miRNA content in the HEK cells after purification is very low (2 to 5 % of the purified small RNA).

miRNA – visualizing synthetic miRNA

To mimic miRNA migration and staining behavior, a mixture containing 80 synthetic RNA oligos with size range of 18-23 nucleotides (from Human miRBase Sequence Database) was run on the 2100 bioanalyzer. Figure 3 shows an electropherogram of 2000 pg of the RNA oligo mix. The calculated size average for the miRNA region is 20 nt which is in good accordance with expected values. The broad peak is due to the range of mobility caused by the different sequences represented in the oligo mix.

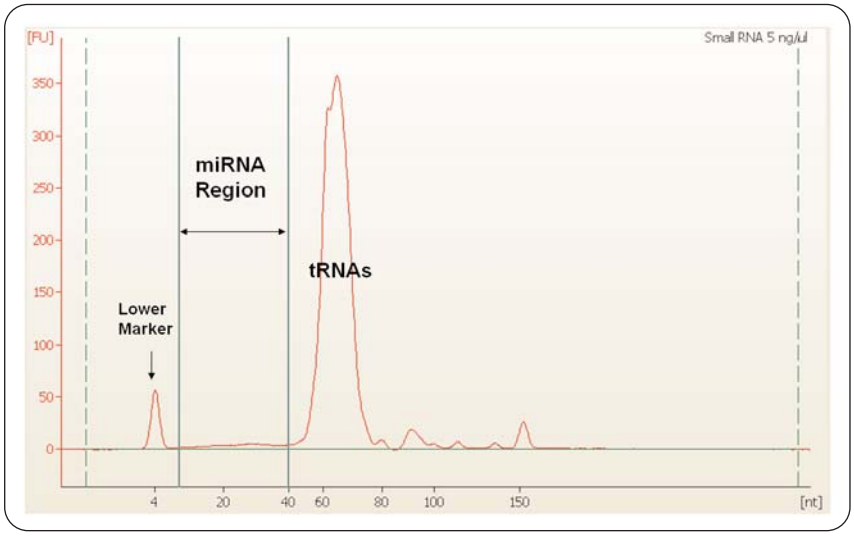


Figure 2
Electropherogram of purified small RNA (5 ng/µL), showing that the major content of the purified fraction is tRNA and indicating the low abundance (2-5 %) of miRNA.

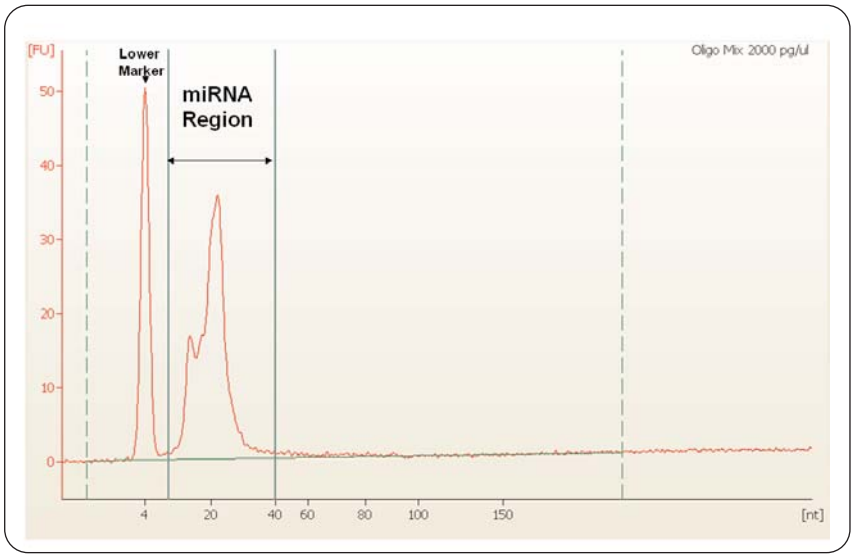


Figure 3
Electropherogram of 2000 pg/µL RNA oligo mix mimicking miRNA migration and staining behavior.

Linear Dynamic Range of Quantification

The Small RNA assay ladder is used as external standard for small RNA quantification. It comprises 6 non-repetitive RNA oligonucleotide sequences as ladder fragments. The fragments sizes ranged from 20 nt to 150 nt and provided intense and sharp peaks (figure 4). To determine the linear dynamic range of miRNA quantification, a master mix of synthetic RNA oligos was diluted from 50 pg/µL to 2000 pg/µL and run on the 2100 bioanalyzer (figure 5). In that range no deviation from linearity could be found, which is in line with the correlation coefficient of 0.999.

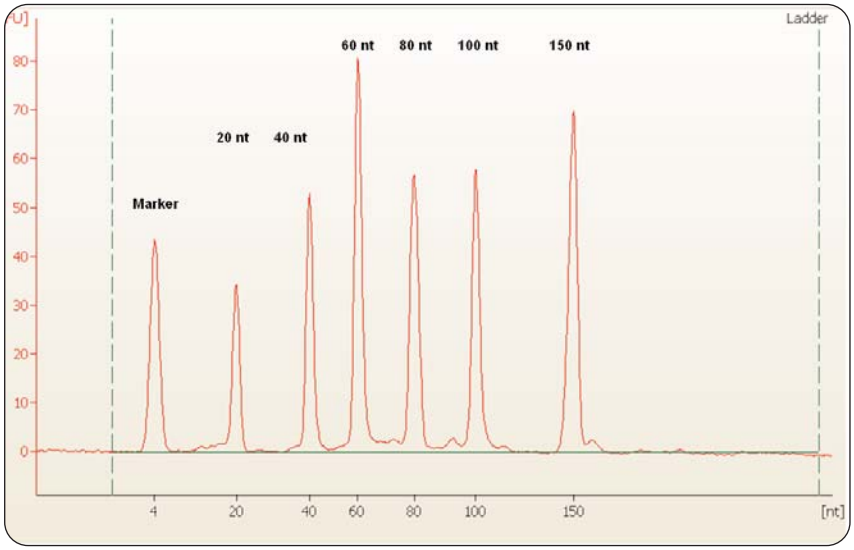


Figure 4
Small RNA ladder electropherogram with 6 bands from 20 to 150 nt.

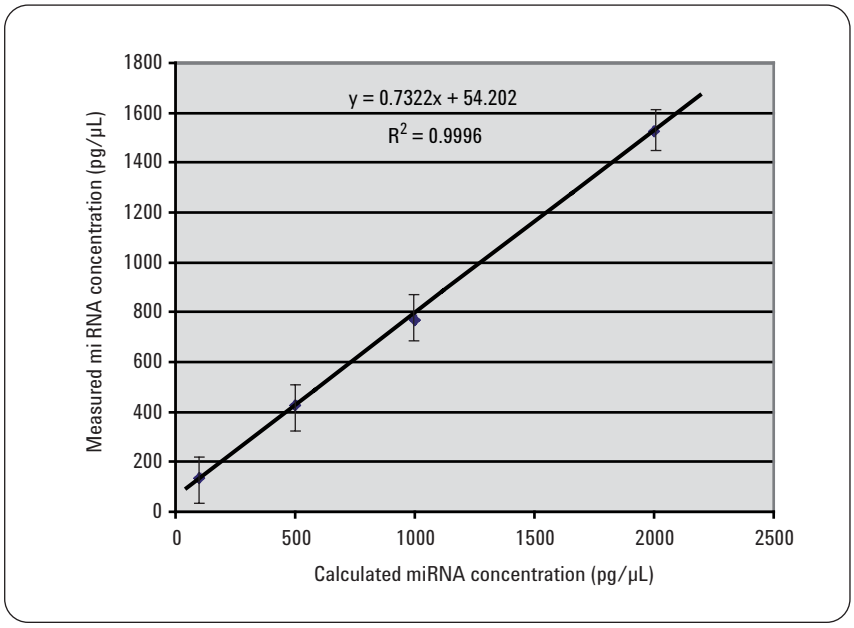
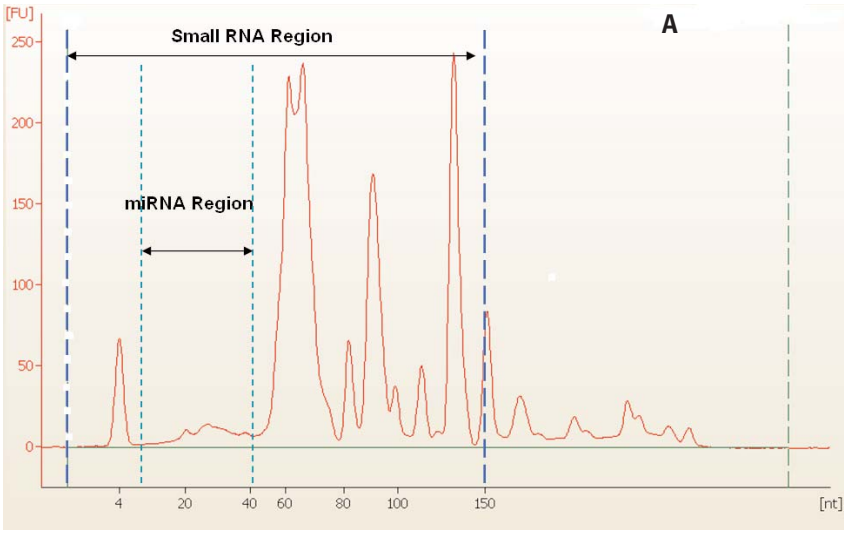


Figure 5
Linear dynamic range of quantification of miRNA detection, detected with an RNA Oligo mix, showing nominal concentration versus concentration determined with the 2100 bioanalyzer.

Input amount of total RNA for miRNA detection

The assay is capable of analyzing pg amounts of miRNA in ng amounts of total RNA. To show this, a sample of mouse thymus total RNA was analyzed in dilution series, starting with a master mix at 150 ng/µL down to 10 ng/µL. For the quantification analysis of total RNA, two regions have been defined. The small RNA region has been reduced to the region spanning from 0 to 150 nt, and the miRNA region from 10 to 40 nt (figure 6 A). Figures 6B and 6C show a plot of the miRNA and small RNA quantification results against the quantity of total RNA loaded. A linear regression resulted in a correlation coefficient of R^2=0.9619 for miRNA and R^2=0.9682 for small RNA. Table D in figure 6 summarizes the average concentration for both regions. The results demonstrate the excellent reproducibility of the miRNA quantification of the Small RNA assay, which is reflected by the CVs well below 14 % across the size range.



D

	Total RNA loaded (ng/µL)	miRNA average Concentration (pg/µL)	Stdv	% CV
miRNA Region	10	231,8	30,7	13,2
	25	522,4	38	7,3
	75	759,4	46,2	6,1
	100	1307,4	114,2	8,7
	150	1731	86,7	5,0

	Total RNA loaded (ng/µL)	Small RNA average Concentration (pg/µL)	Stdv	% CV
Small RNA Region	10	3223,1	193,5	6,0
	25	6802,2	377,85	5,6
	75	10636,6	1387	13,0
	100	17676,1	1460,22	8,3
	150	22630,5	2037,9	9,0

Figure 6
Quantification of small RNA and miRNA contents of a total RNA sample.
Mouse thymus total RNA was analyzed in 5 different concentrations. The plots of the small RNA and miRNA quantification versus the quantity of total RNA loaded (B and C respectively) show the linear dynamic range of quantification of the Small RNA assay for both regions. The quantification results are summarized in table D.

Sensitivity

To evaluate the sensitivity of the Small RNA assay a dilution of one in ten of the small RNA ladder in pure water was analyzed. At this dilution level, the 40 nt fragment for example had a concentration of 50 pg/µL. Figure 7 shows that even at this low concentration level, the 40 nt fragment was detected with a signal to noise ratio greater than 3. High ionic strength buffer will reduce signal intensity. Effect on the quantification are corrected by a new sophisticated algorithm in the software, using the lower marker as an internal reference.

Small RNA Assay specifications

Figure 8 shows the analytical specifications of the Small RNA assay and the recommended total sample concentrations for different sample types (total RNA, enriched small RNA and oligonucleotides).

Conclusion

The Small RNA assay used with the Agilent 2100 bioanalyzer provides a solution to accelerate identification and characterization of small RNA molecules.

The assay is fast, highly sensitive and offers a new tool for visualization and quantification of small RNA. It allows researchers to identify and monitor small nucleic acid samples in total RNA extracts, or optimize small RNA extraction quality.

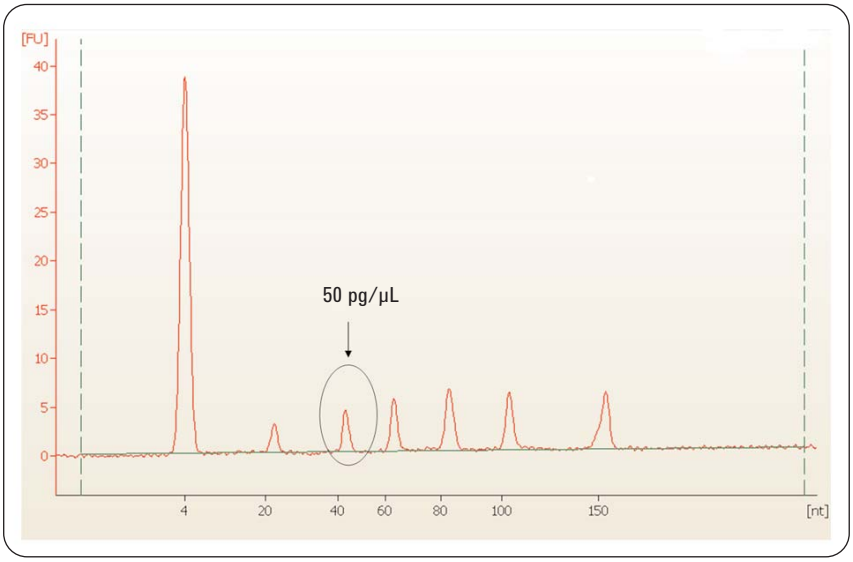


Figure 7
Small RNA Assay sensitivity: highlighting the detection of the 40 nt ladder fragment at a concentration of 50 pg/µL.

Physical Specifications	
Type	**Specifications**
Analysis run time	30 minutes
Number of samples	11 samples/chip
Sample volume	1 µL
Assay kit stability	4 months (Storage temperature see individual box!)

Analytical Specifications	
Specification	**Small RNA Assay**
Analysis range	6-150 nt
Quantitation reproducibility	25 % CV (for ladder as an example)
Quantitative Range	50-2000 pg/µLfor purified miRNA (in water)
Max. Buffer concentration*	10 mM Tris and 0.1 mM EDTA
Sensitivity (Signal/Noise > 3)	50 pg/µL(for 40 nt fragment of diluted ladder)

*) Due to the high sensitivity of the assay, different ions and higher salt concentrations might influence the performance of the assay.

Recommended Concentration	
Sample type	**Concentration**
Total RNA	1-100 ng/µL
Enriched Small RNA*	1-20 ng/µL
Oligonucleotides	100 to 2000 pg/µL

*) For example by commercial purification kit or preparative gel extraction.

Figure 8
Small RNA assay specifications.

References

1.
"Cancer Genomics: Small RNAs with big impacts" *Nature 435*, *745-746*, *June* **2005.**

2.
"MicroRNAs in Gene Regulation: When the Smallest Governs it all." *Journal of Biomedecine and Biotechnology*, *206*, *pages 1-20*, **2006.**

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