Successful analysis of low RNA concentrations with the Agilent 2100 bioanalyzer and the RNA 6000 Pico LabChip® kit

Technical Overview

Introduction

Since its introduction, the RNA 6000 Pico LabChip® kit has become a valuable addition to the existing LabChip kit series. Although not designed as a quantitative assay, its high sensitivity, down to picogram levels, allows a fast, automated analysis and quality control of low concentrated nucleic acid samples. Samples to be analyzed include single stranded molecules derived from microdissected tissues or needle biopsies, and cDNA. Due to the kit's high sensitivity, careful sample and chip preparation is mandatory for satisfactory results. This Technical Overview summarizes the different factors that influence the performance of the RNA 6000 Pico assay.

Essential measurement practices

During assay preparation the guidelines regarding reagent preparation and instrument maintenance must be strictly followed. Important technical points are summarized in the supporting documentation found in the kit manual in the essential measurement practices section. The cleaning procedures before and after the chip run must be performed accurately. It is strongly recommended to use a dedicated electrode cartridge exclusively for the RNA Pico assay. This helps to prevent any DNA or protein cross- contamination that may disturb the RNA analysis. If different work groups share a Agilent 2100 bioanalyzer instrument, it is recommended that each group use their own set of consumables and electrode cartridge. Additional cartridges can be ordered as spare parts (part number 5065-4413). To clean the electrode pins, RNase-free water is sufficient in most cases. Before starting the chip run, it is important that the electrodes of the cartridge are completely dry, otherwise leak currents may disturb the analysis. A more thorough cleaning with

RNaseZAP (Ambion, Inc. cat. No. 9780) is required in case of RNase contamination. In any case, residual RNaseZAP must be removed completely by thorough rinsing of the electrodes with RNase-free water. In general, it is important to carefully follow the cleaning guidelines described in the reagent kit guides. Details of the maintenance procedures can be found in the Online Help of the 2100 bioanalyzer software as well as in the maintenance part of the "2100 How-to-Use" multimedia CD-ROM (part number G2946-60002).

When the chip is prepared, samples are mixed with marker solution during a vortexing step. After the vortexing step, the user should check for liquid spills between the wells on the chip. This may cause leak currents between the wells of the chip, which lead to failed chip runs. The RNA Pico dye concentrate contains low levels of detergent that reduce the surface tension of the gel-dye mix. After the vortexing step, the user should check for liquid spill on top of the chip caddy. In most cases, the spill is visible as a very thin film of liquid only and no individual droplets can be seen. As the complete removal of the spill is not always feasible, it is recommended to reduce the vortex speed from 2400 rpm down to 2000 rpm.

RNA isolation kit

The compatibility of the RNA 6000 Pico kit with commercially available and widely used RNA isolation kits (table 1) was investigated. Most of the kits use a spin-column based approach to bind, wash and elute the RNA. Each kit differs in column material and in included reagents. Because the Pico kit is known to be sensitive to salt, variations of its performance can be expected. Differences in the ionic strength of the ladder and the sample will be reflected in a quantitation offset. During assay preparation, the RNA ladder (Ambion, Inc. cat. No. 7152) is diluted with de-ionized RNase-free water. The higher the ionic strength of the sample, the more inorganic ions are injected into the separation channel instead of charged RNA molecules. In extreme cases, it was observed that ribosomal bands disappear, especially the 28S band (figure 1). This may lead to the wrong conclusion that the RNA isolation failed or the RNA sample is degraded. To calculate the concentration of the sample, the software determines the area under the entire RNA electropherogram. The ladder, which provides a concentration/area ratio, is applied to transform the sample area into concentration

Kit	Manufacturer
Total RNA Isolation Mini Kit[2]	Agilent Technologies
PicoPure®, RNA Isolation Kit	Arcturus
RNAqueous®, Micro Kit	Ambion
RNeasy®, Mini Kit	QIAGEN
Absolutely RNA®, Nanoprep Kit	Stratagene
Purescript®, RNA Kit	Gentra Systems
RNA Extraction Kit	PALM

Table 1
RNA isolation kits, tested for the compatibility with the Agilent RNA 6000 Pico LabChip kit.

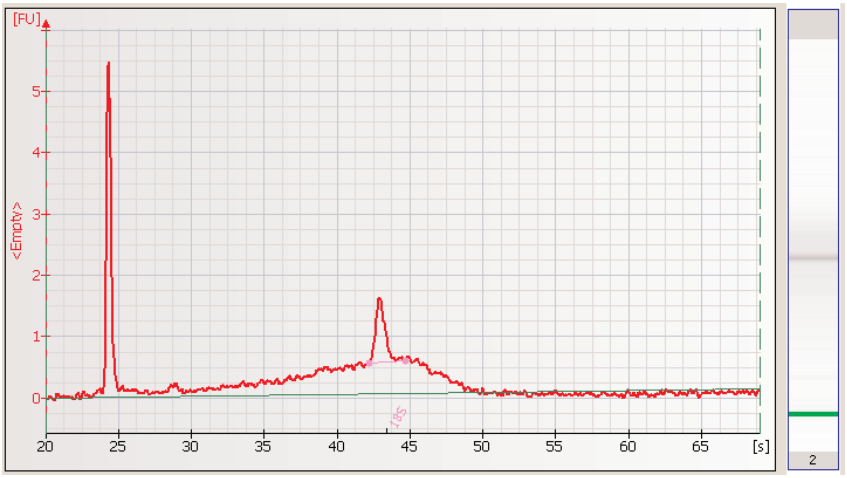

Figure 1
Electropherogram of a total RNA sample isolated after laser capture microdissection. The 28S band is not injected because of the high ionic strength of the sample.

values. As a consequence, a different salt content in the ladder and the sample will lead to a quantitation offset. Thus, it is recommended to dissolve or elute the RNA sample in de-ionized water at the end of the isolation process.

In a validation study, elutant from mock-isolations were produced according to the manufacturer's protocol. Commercially available standard total RNA or messenger RNA samples were diluted with the mock-eluates. RNA diluted with de-ionized water was used as a reference sample. All chips were prepared according to the instructions provided with the RNA 6000 Pico LabChip kit. The majority of RNA isolation kits had no negative impact on the performance of the kit. Among the different kits tested, the RNA concentration determined with the Pico kit varied by a factor of 4 (mean value of 6 or 12 mock-isolations). It should be noted that not the performance of the isolation kit was tested but the influence of the different elution buffers and matrix components on the Pico kit. The validation test confirmed the expected differences. Total RNA samples (500 pg/µL) prepared with mock-eluates of the Stratagene "Absolutely RNA Nanoprep-Kit" showed very low signal intensities (figure 2). Compared to RNA reference sample that was prepared by a dilution with de-ionized water (figure 2, red electropherogram), a signal decrease by a factor of 10 can be observed. When using this kit, the specified lower limit of detection for the RNA Pico kit of 200 pg/µL can thus not be guaranteed. Results from an in-depth analysis

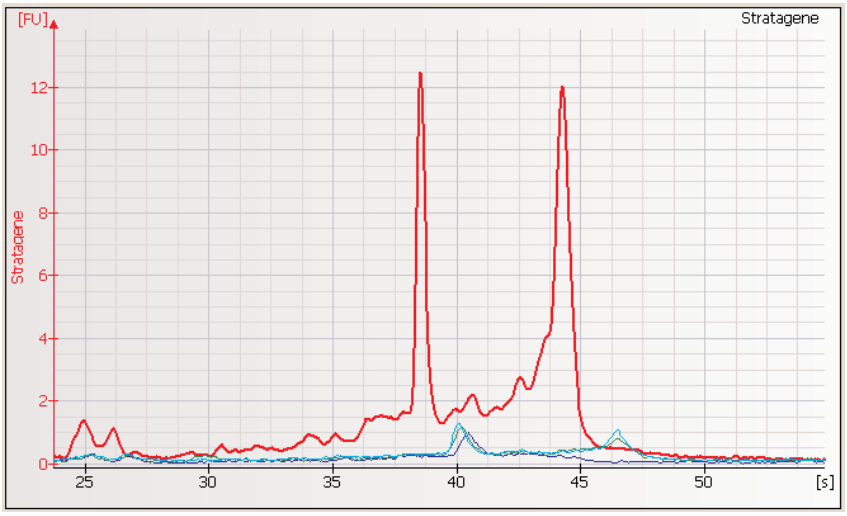

Figure 2
Quality control of total RNA samples diluted with mock-elutants of the Stratagene "Absolutely RNA Nanoprep-Kit" using the RNA 6000 Pico kit. Red electropherogram: 500 pg/µL total RNA sample prepared by dilution of a stock solution with de-ionized water. Other electropherograms: 500 pg/µL total RNA sample prepared by dilution of a RNA stock solution with mock-elutants.

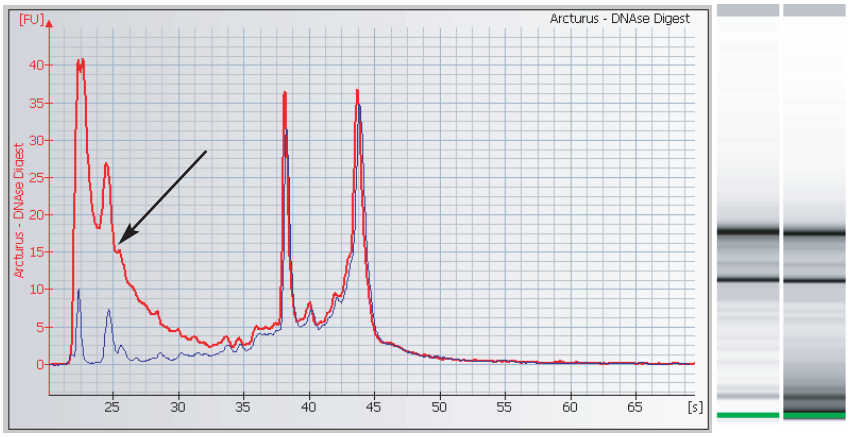

Figure 3
Gel like image and overlay of 2 electropherograms of total RNA samples generated with the mock-eluates of the Arcturus "PicoPure-Kit". Total RNA sample before (red) and after (blue) an on-column DNase digestion.

confirmed that matrix components of this kit lead to incomplete sample injection into the separation channel of the microfluidic chip. The Arcturus "PicoPure-Kit" contains DNA as a precipitation-carrier, which is visible in the electropherogram as well as in the gel-like image (figure 3). Because the DNA interferes with the quantitation, an on-column DNase digestion according to the manufacturer's protocol is recommended to remove the contamination.

In order to successfully analyze RNA isolated with the Gentra Systems “Purescript RNA-Kit”, a protocol modification is recommended. After the RNA precipitation, the RNA hydration should be done with de-ionized RNase-free water, instead of the supplied RNA hydration solution that significantly reduces the sensitivity of the RNA 6000 Pico kit.

Salt concentration

In a second validation study, the compatibility of the RNA 6000 Pico kit with different ions was tested. In general, the presence of ions negatively affects the performance of the RNA 6000 Pico kit. The higher the ionic strength of the sample, the more inorganic ions are injected into the separation channel instead of charged RNA molecules. This leads to a loss of sensitivity of the assay. Figure 4 confirms that not only the sensitivity is affected. The presence of salt also can affect the migration times of the ribosomal fragments. Among the salts tested, differences can be detected. $MgCl_2$ shows a strong impact on the sensitivity of the assay, while NH_4Ac shows only a moderate effect. In general, the salt concentration should be kept as low as possible. The best assay performance can be ensured if the RNA samples are in de-ionized water.

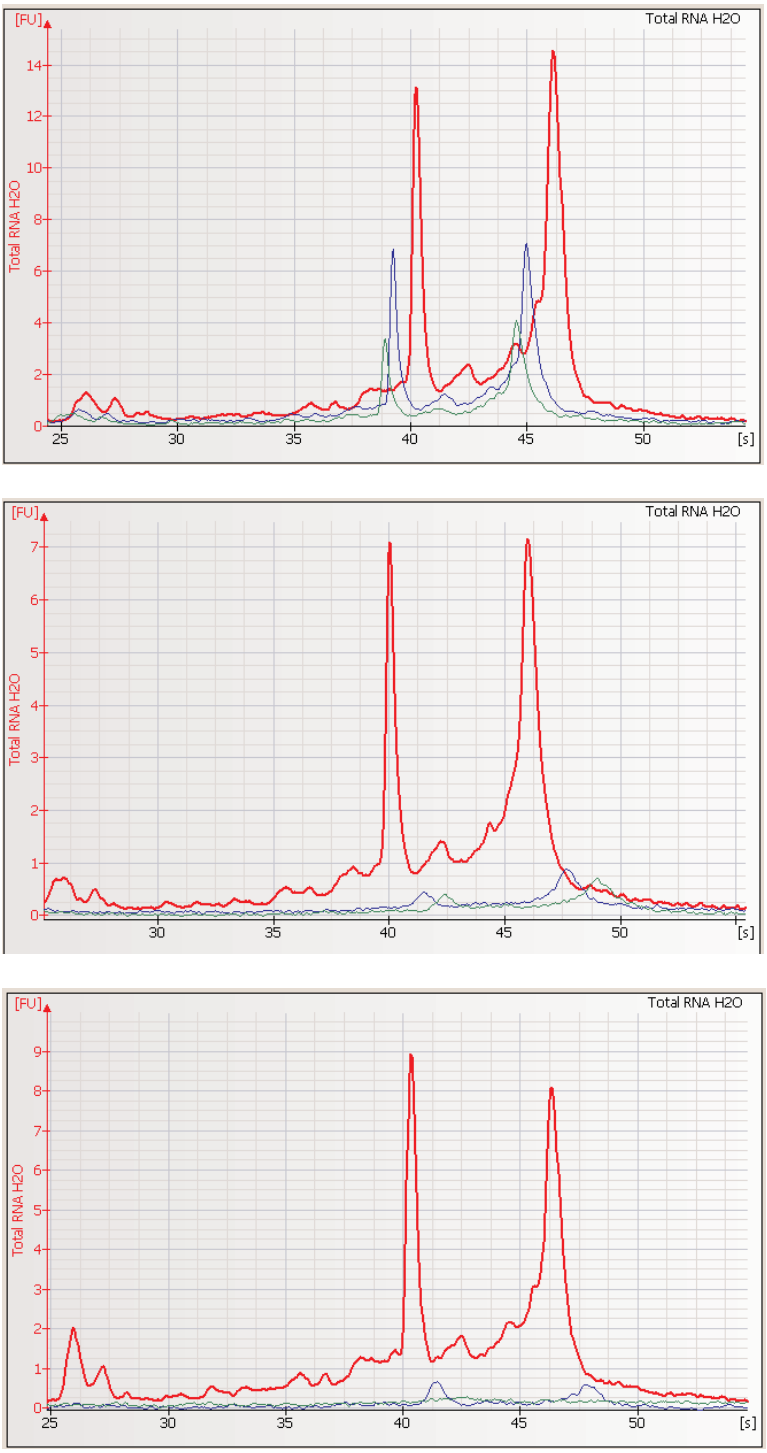

Figure 4
Effect of increasing salt concentration on the analysis of 500 pg/µL total RNA. Red, blue and green electropherogram corresponds to 0 mM, 5 mM and 10 mM salt, respectively. A) NH_4Ac, B) NaCl, C) $MgCl_2$.

Pipette tips

In addition to the type of RNA isolation kit or the ionic strength of the RNA sample, the type of pipette tips plays a critical role in assay performance. Figure 5 demonstrates baseline fluctuations that are caused when using ART® brand tips. Aerosol resistant tips are used to prevent cross contamination of aerosols from sample to sample. This may ultimatively lead to failed chip runs. Because no sample was loaded on the chip, only the lower marker fragments are visible in the electropherograms. If baseline fluctuations are observed with the RNA 6000 Pico kitkit, it is recommended to check the filter tips as a possible cause of the problem. In these cases, a switch to a non-aerosol resistant pipette tip should be made. The reason for the observed baseline abnormalities is not yet fully understood.

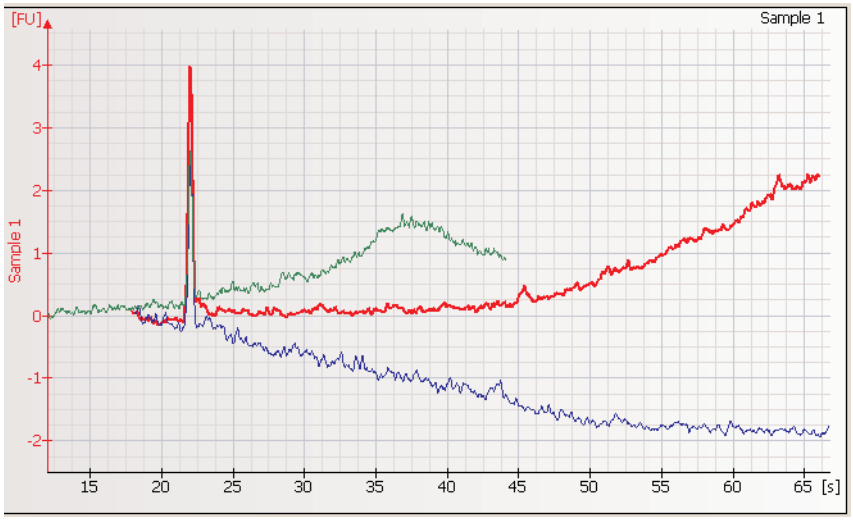

Figure 5
RNA 6000 Pico Assay prepared with ART® (aerosol resistant tips) filter tips (Molecular Bio Products). No sample was loaded. Baseline fluctuations are visible in 3 of the 11 electropherograms.

Conclusion

In two validation studies the influence of different RNA isolation kits, ions and salt concentrations on the performance of the RNA 6000 Pico kit was investigated. Differences in the ion strength of ladder and sample strongly affected the sensitivity of the kit. It should be noted that the RNA 6000 Pico kit was not developed to be a quantitative assay. Concentrations delivered by the software should be always regarded as concentration estimations. Because the RNA 6000 Pico assay is a high sensitivity assay, the essential measurement practices must be closely followed to obtain satisfactory results.

References

1.
"High sensitivity quality control of RNA samples using the RNA 6000 Pico LabChip kit", *Agilent Application Note*, *Publication Number 5988-8554EN*, **2002.**

2.
Efficient method for isolation of high quality concentrated cellular RNA with extremely low levels of genomic DNA contamination, *Agilent Application Note*, *Publication Number 5989-0322EN*, **2002.**

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Published March 1, 2004
Publication Number 5989-0712EN

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