Analysis of Cy5-labeled cRNAs and cDNAs using the Agilent 2100 Bioanalyzer and the RNA 6000 LabChip® kit

Application Note

Life Sciences

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Introduction

Enormous progress is being made in the cloning, mapping, and sequencing of various organisms, and the Human Genome Project is soon to be completed. However, the function, expression and regulation of many genes are still unknown. The next research phase will therefore focus on understanding the role of specific genes. One approach for exploring the role of a gene is to study its expression pattern. A variety of methods are available for the detection and quantitation of gene expression levels, including northern blotting, differential display, nuclease protection assays, sequencing of cDNA libraries, serial analysis of gene expression (SAGE), and the quickly growing field of microarray technology. Oligonucleotide and cDNA microarrays are anticipated to become a widespread application. In addition to expression analysis, microarrays can also be applied to genotyping and mutation screening. Many of these applications use fluorescently labeled probes, such as Cy5- and Cy3-labeled cRNAs and cDNAs. Labeled cRNAs and cDNAs are generated by incorporating fluorescently labeled nucleotides during T7 transcription and reverse transcription reactions. The quality/integrity of the RNA that is used to synthesize labeled cDNAs or cRNAs as well as the success of the labeling reaction itself are critical factors for the performance of microarrays.

With the increased interest in expression profiling, genotyping and mutation detection applications, there has been a steadily growing demand for faster, more automated tools for DNA and RNA analysis. The Agilent 2100 bioanalyzer utilizing the LabChip technology from Caliper Technologies Corp. provides a compact system for the rapid analysis of nucleic acids, integrating multiple experimental steps. The Agilent 2100 bioanalyzer can be used with the RNA 6000 LabChip kit to measure both the concentration and the quality/integrity of total and mRNA samples as described in two other application notes (Agilent publication numbers 5968-7493E and 5968-7495E). Furthermore, this application note shows that the kit can also be used to analyze Cy5-labeled cRNAs and cDNAs before hybridizing these samples to microarrays. This offers several advantages over the current gel-based techniques. These include

- minimal sample consumption
- reduced analysis time and manual intervention
- enhanced separation performance
- excellent reproducibility

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Experiment

All chip-based separations were performed on the Agilent 2100 bioanalyzer in combination with the RNA 6000 LabChip kit and the dedicated assay for the analysis of Cy5-labeled nucleic acids. This application note describes the use of two different experimental protocols for the analysis of Cy5-labeled cRNAs or cDNAs.

Analysis of Cy5-labeled cRNAs

All chips were prepared according to the chip preparation protocol provided with the RNA 6000 LabChip kit.

Analysis of Cy5-labeled cDNAs

For the analysis of Cy5-labeled cDNAs, the chip preparation protocol provided with the RNA 6000 LabChip kit was modified. For this adapted protocol, Cy5-dCTP (Amersham Pharmacia Biotech Inc.) is added to the gel matrix and the sample buffer for focussing. The gel-dye mix was prepared by filtering 400 µl of the gel matrix through a spin filter (supplied with the RNA 6000 kit) for 10 minutes at RT and 10 µl Cy5-dCTP (200 nM in Tris-HCl pH 7.4) are added to a final concentration of 4.9 nM. In this experiment, the RNA 6000 dye is not added to the gel matrix. In addition, 4.9 nM Cy5-dCTP was also added to the sample buffer. The channels of the RNA chip were filled with the gel-dye mixture. Then, 5 µl of the sample buffer/dye mixture was added to each sample well and 6 µl to the ladder well. 12 samples (1 µl each) were loaded into the sample wells of the chip. No sample or RNA 6000 ladder was pipetted into the ladder well. The chip was then vortexed and run on the Agilent 2100 bioanalyzer with the dedicated assay for the analysis of Cy5-labeled nucleic acids.
The cDNA labeling reaction is usually followed by an RNA degradation step, such as base hydrolysis or RNase digestion. Analysis of RNase-containing samples with the Agilent 2100 bioanalyzer can result in contamination of the electrodes by RNase. Therefore, the decontamination of the electrodes with RNase ZAP (Ambion Inc.) and the cleaning chips (provided with the RNase 6000 LabChip kit) following each experiment is necessary.

Results

Analysis of Cy5-labeled cRNAs

The RNA 6000 LabChip kit was used with the Agilent 2100 bioanalyzer to analyze Cy5-labeled cRNA samples generated from mouse liver poly (A)+ RNA. 1 µl of Cy5-labeled cRNA can be analyzed in the concentration range from 10 to 500 ng RNA/µl as determined by UV-visible spectroscopy. The total sample area is displayed along with the electropherogram and multiple samples can be overlaid for comparison. Figure 1 shows the analysis of unlabeled and Cy5-labeled cRNA. Unlabeled cRNA can be detected with the RNA 6000 dye. The Cy5-labeled samples generate a significantly stronger signal due to the additional fluorescence emitted by the Cy5-label. The RNA 6000 ladder can be employed to estimate the size range of the labeled product. As shown in figure 1, the Cy5-labeled cRNA ranges in size between 500 and 2000 bases.

Analysis of Cy5-labeled cDNAs

Cy5-labeled cDNA was generated from total RNA from ML-1 cells by reverse transcription in the presence of 25 µM Cy5-dCTP. The samples were treated with RNase, column-purified and concentrated. To evaluate the performance of the Agilent 2100 bioanalyzer, Cy5-labeled cDNA and failed labeling reactions due to modifications of

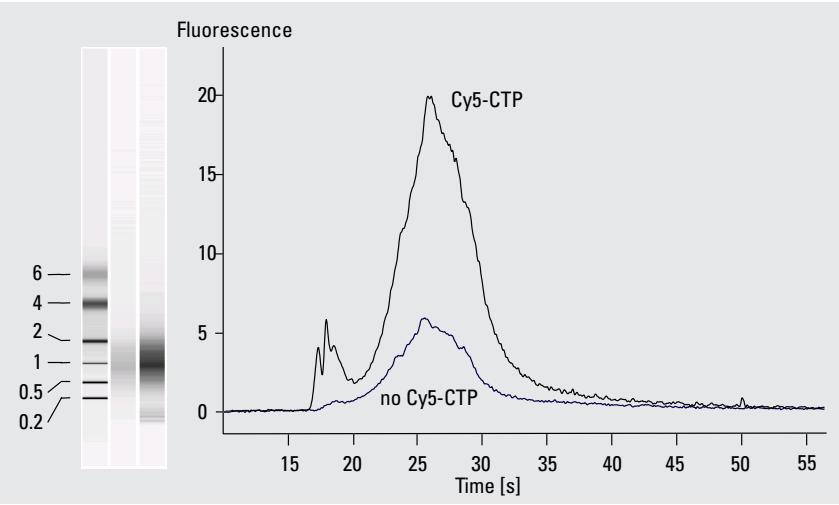

Figure 1
Electrophoretic separation of 100 ng/µl unlabeled cRNA and 100 ng/µl Cy5-labeled cRNA using the RNA 6000 LabChip kit. The custom-made RNA 6000 ladder (150 ng/µl, Ambion Inc. Cat # 7152) was also separated on the chip. Fragment sizes are given in kb. Data is displayed as a gel-like image and as overlaid electropherograms.

the labeling protocol were analyzed using the RNA 6000 LabChip kit (figure 2-A) and compared to the analysis of the same samples by denaturing gel electrophoresis (figure 2-B). The analysis of Cy5-labeled cDNA with conventional slab gel electrophoresis resulted in the separation of the cDNA as a very broad smear. Not all failed labeling reactions could be identified with certainty. The analysis of the same samples with the Agilent 2100 bioanalyzer also separated the cDNA into a smear, but allowed the easier identification of failed labeling reactions. Additional bands can be identified on the gel-like image (figure 2-A), which can not be seen on the gel (figure 2-B). In addition, the chip-based analysis allows the user to separate free dye from the labeled cDNA, which is visible as a sharp band in the gel-like image. cDNA generated with the normal labeling protocol (25 µM Cy5-dCTP) and in the presence of a 6-fold higher concentration of Cy5-dCTP does not show a distinctively different pattern on the gel (figure 2-B). However, more details can be derived from overlaying the two electropherograms (figure 2-C). The high Cy5-dCTP concentration resulted in a lower cDNA labeling efficiency compared to the normal protocol. This can be explained by the inhibition of the labeling reaction by high dye concentrations (unpublished data). While both the chip-based cDNA analysis and gel electrophoresis techniques require the same sample amount (1 µl), the analysis time and manual intervention is greatly reduced by the chip-base method. Additional advantages also include the data analysis and comparison functions which are integrated in the system.

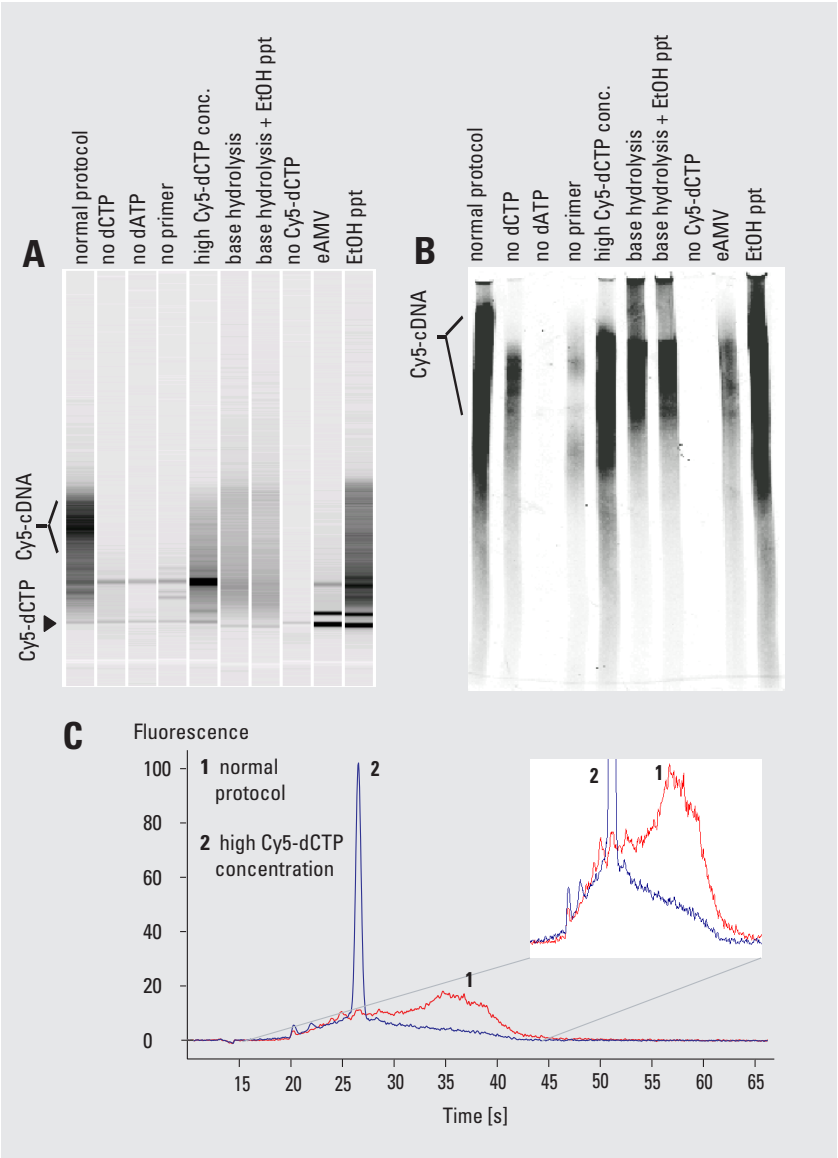

Figure 2
Cy5-labeled cDNA (lane 1) and several failed labeling reactions (lane 2-10) were analyzed with the Agilent 2100 bioanalyzer (A). For comparison, the same samples were separated on a denaturing acrylamide gel, (B). Cy5-labeled samples in denaturing loading buffer (90% formamide, 1x TBE and a trace of bromophenol blue) were electrophoretically separated on 6% TBE urea gels (NOVEX) at 200 V for about 45 min. The image was obtained using the Molecular Dynamics Storm fluorescence imager. The electropherograms of two of the samples analyzed with the Agilent 2100 bioanalyzer, as shown in A were compared by employing the overlay function embedded in the bioanalyzer software (C). The inset shows the same electropherogram enlarged for better comparison.

Conclusion

The Agilent 2100 bioanalyzer is designed for the rapid analysis of nucleic acids. The use of Lab-on-a-Chip technology allows the analysis of multiple samples through a single separation channel with excellent reproducibility of the system. Separation performance is superior to gel electrophoresis and analysis time is greatly reduced. The Agilent 2100 bioanalyzer can be used to analyze Cy5-labeled cRNAs and cDNAs before hybridization to expensive microarrays. The chip-based analysis allows the comparison of various labeled samples according to their separation pattern and peak area. In addition, a rough size estimate can be obtained and failed cDNA labeling reactions can be identified.

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