

Optimizing cRNA fragmentation for microarray experiments using the Agilent 2100 bioanalyzer

Application

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Introduction

Oligonucleotide arrays are a powerful tool for gene expression studies. One experiment can reveal the concurrent expression patterns of thousands of relevant genes. Oligo arrays typically use fluorescently labeled amplified RNA (cRNA) generated by T7 transcription as targets. One parameter affecting the performance of oligo arrays is the quality of the cRNA targets that are hybridized to them. However, before using the cRNA in hybridizations, researchers often forgo sample quality checks during their generation because of issues concerning time, cost and excessive sample consumption. The Agilent 2100 bioanalyzer and RNA 6000 LabChip[®] kit provide a simple, rapid way to qualify microarray targets throughout their creation and before hybridization, thus raising the potential for successful microarray results.

Successfully amplified cRNA ranges in length from 50–3000 bases. The larger transcripts often contain secondary structure, which can interfere with hybridization and increase the opportunity for non-specific cross-hybridization to the 20–70 base long probe oligos attached to the array. Labeled cRNA makes a better target for oligo arrays once it has been fragmented to an optimal size of 50–200 bases long. The structures of the fragmented targets are less complex, which helps improve their specificity and raises the average feature signal intensity on the microarray. Array performance is directly related to the amount of cRNA fragmentation. Fragmentation of the cRNA should be monitored to determine when a majority of the sample is of the length for optimal microarray performance.

The Agilent 2100 bioanalyzer can be used to easily optimize and monitor cRNA fragmentation reactions and is faster and more sensitive than gel electrophoresis. The small benchtop system uses LabChip[®] technology to integrate sample separation, detection, and data analysis to determine the concentration and integrity of 12 samples in a total analysis time of about 25 minutes. This application note outlines the creation workflow of fluorescence labeled cRNA from HeLa RNA. The Agilent 2100 bioanalyzer is used to follow the cRNA sample generation and to optimize the fragmentation. In correlation, the microarray data collected from this sample is presented and interpreted in relation to the data collected from the bioanalyzer.



Agilent Technologies



Materials and methods

Human HeLa cell total RNA and human HeLa cell (S3) poly A+ RNA were purchased from Clontech Laboratories, Inc. (Palo Alto, CA). The RNA 6000 ladder was procured from Ambion Inc. (Austin, TX). The mRNA was amplified and fluorescently labeled with Cy3 and Cy5 using the Fluorescent Linear Amplification Kit from Agilent Technologies (Palo Alto, CA), part number G2556A.

This kit uses proprietary technology to convert and amplify mRNA species to fluorescently labeled complementary RNA with maximum performance and sensitivity. The labeled samples were prepared according to kit protocols. Samples prepared for the cRNA fragmentation optimization were incubated at 60 °C in the presence of 10 mM zinc acetate, for varying lengths of time and quenched using an excess of EDTA.

All total RNA, cRNA and fragmented cRNA samples were analyzed on the Agilent 2100 bioanalyzer using the eukaryote total RNA assay and RNA 6000 LabChip kits supplied by Agilent Technologies. The mRNA samples were run using the mRNA assay. The fragmented cRNA optimization samples were additionally run under non-denaturing conditions on Reliant 1 % agarose precast gels from Amresco (Solon, OH) stained with SYBR Gold nucleic acid stain obtained from Molecular Probes, Inc. (Eugene, OR).

Results and discussion

1. Quality analysis of total and messenger RNA

The first step in the target generation process is a quality assessment of the template RNA to determine that the sample has not been degraded or contaminated. Agarose gel electrophoresis can be used to monitor the quality of RNA preparations, but a large amount of sample is required to perform the lengthy analyses involved. In contrast, the bioanalyzer eliminates these two deterrents, requiring only 25 ng of RNA sample and 30 minutes for the analysis.

The bioanalyzer electropherogram of total RNA shows two distinct ribosomal peaks corresponding to the 18S and 28S ribosomes, and a relatively flat baseline between the 5S and 18S ribosomal peaks (figure 1). In contrast, degraded total RNA will lack a smooth baseline, and typically contains multiple peaks that are as large or larger than the ribosomal peaks.

Some cRNA procedures utilize purified poly A messenger RNA as the template in the reverse transcription reaction step of target creation. Figure 2 shows the analysis of HeLa mRNA. Messenger RNA should be checked for

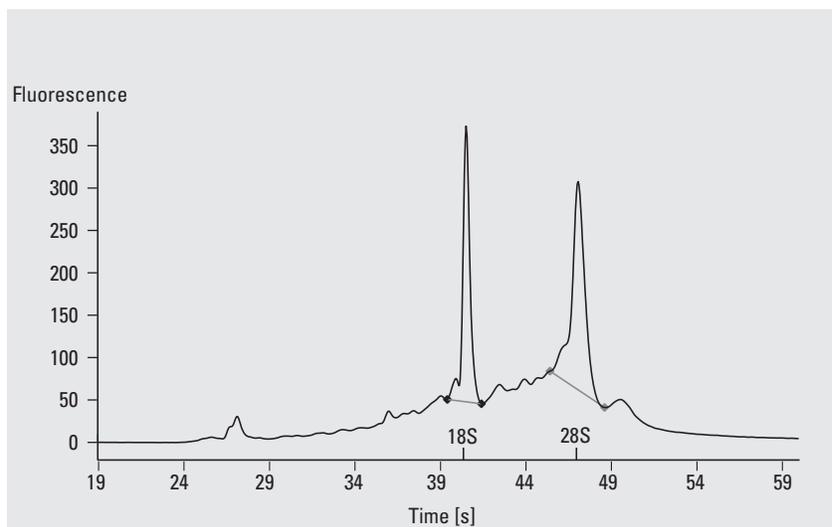


Figure 1
Electropherogram of high quality HeLa cell total RNA (500ng/μl) purchased from Clontech Inc. (California, USA). The sample was analyzed using the RNA 6000 LabChip kit and the eukaryote total RNA assay.

degradation as well as significant levels of ribosomal RNA contamination. Too much ribosomal contamination can artificially inflate the measured concentration of starting mRNA template. Lowered mRNA starting template could lead to poor results later in the experiment. The Agilent 2100 bioanalyzer automatically calculates the percent ribosomal RNA in messenger RNA samples, and can detect as low as five percent contamination.

2. Creation of cDNA and labeled cRNA

Labeled cRNA is made in two sequential reactions. There is no amplification involved in the first reaction, which generates unlabeled, double stranded cDNA through reverse transcription. A quality check of the cDNA is normally not performed due to the limited amounts of cDNA produced. The entire amount is used as the template in the next reaction to synthesize single stranded, fluorescently labeled cRNA.

Only 1–2 μ l of the purified cRNA, generated by T7 amplification, is needed for analysis on the bioanalyzer using the RNA 6000 LabChip kit and the total RNA assay. This is done before proceeding on to fragmentation and hybridization to ensure that the cRNA amplification was successful. An electropherogram of a good cRNA reaction will consist of a smear of products falling between 50 and 3000 bases long (figure 3). A feature that often appears in the electropherograms of Cy5 labeled

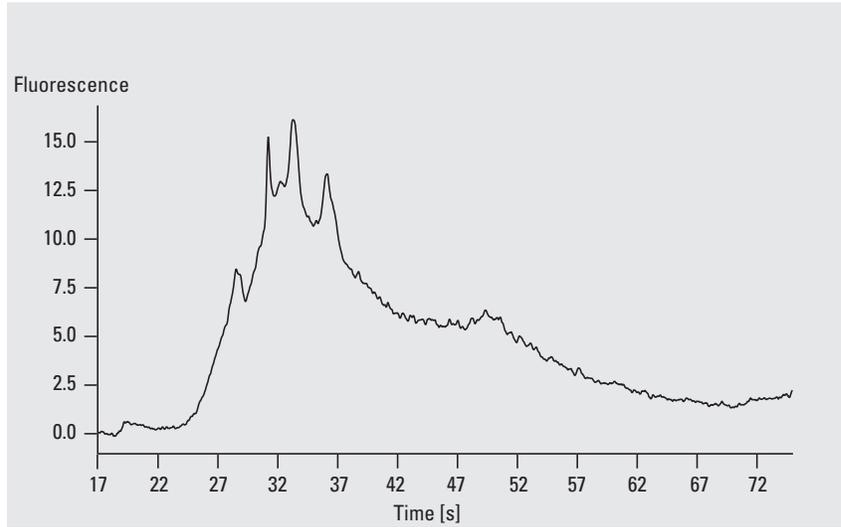


Figure 2
Electropherogram of high quality HeLa cell (S3) poly A+ mRNA (200ng/ μ l) purchased from Clontech Inc. (California, USA). The sample was analyzed using the RNA 6000 LabChip kit and the mRNA assay.

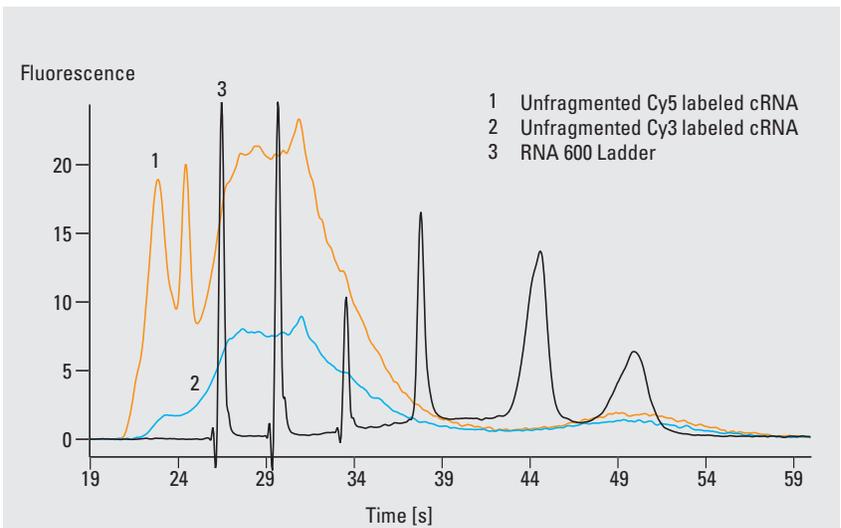


Figure 3
Overlaid electropherograms of Cy3 and Cy5 labeled HeLa cRNA samples and the RNA 6000 ladder with fragment sizes of 200, 500, 1000, 2000, 4000, and 6000 bases. The distribution of the cRNA samples extends to approximately 2000 bases long, with the greatest sample abundance between 200 and 700 bases in length. The two strong peaks in the Cy5 labeled sample at 22 and 24 seconds corresponds to free Cy5-NTP not removed during the purification.

cRNA samples is a strong peak at approximately 22 seconds which corresponds to free Cy5-NTP not removed during the purification. On average, the Cy5 peak height and the cRNA sample peak height are equal. Small amounts of free dye in the sample will not affect microarray performance, however if the Cy5 peak is more than two fold higher than the cRNA sample peak, this may indicate a failed reaction which would result in a high fluorescence background on the microarray.

3. Analysis of the cRNA fragmentation time course

Before hybridization to a microarray, the cRNA should be fragmented to sizes ranging from 50–200 bases long to improve target specificity. Multiple fragmentation techniques exist, however, exposure to zinc acetate is a very effective method because it is quick and easy to control. The relationship between cRNA sample exposure to zinc acetate and average sample length can be graphically displayed by overlaying the electropherograms of multiple samples along a fragmentation time course (figure 4). At the 10-minute time point, the cRNA sample is still distributed over a relatively broad size range. With each successive time point, the amount of longer fragments is diminished, while the distribution of the cRNA fragments becomes more concentrated at shorter fragment sizes. At the 30 and 40 minute time points, the majority of the sample forms a peak that migrates at 23–25 seconds. At this time, a majority of

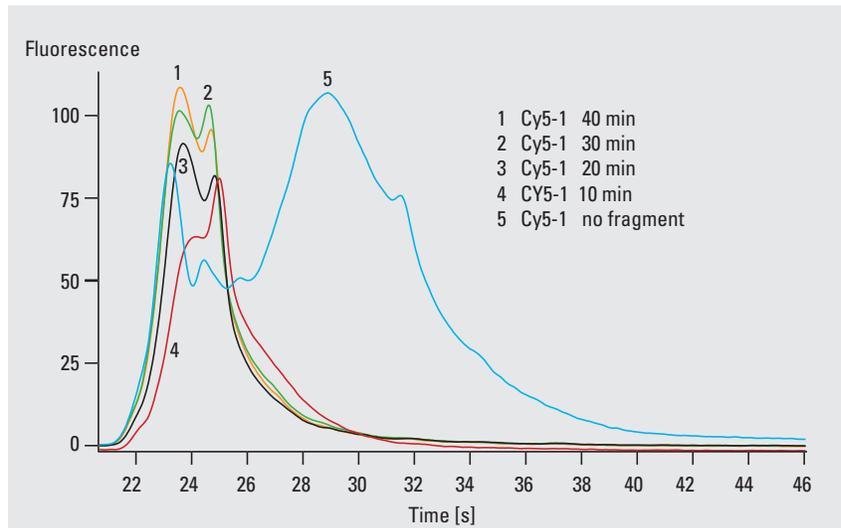


Figure 4
Overlaid electropherograms from a Cy5 labeled HeLa cRNA fragmentation time course. The cRNA was fragmented at 60 °C with a buffer containing 10mM zinc. The fragmentation reactions were stopped at 0, 10, 20, 30, and 40 minute time intervals. 1 µl of fragmented cRNA sample from each time point was analyzed on the bioanalyzer using the RNA 6000 LabChip kit and total RNA assay.

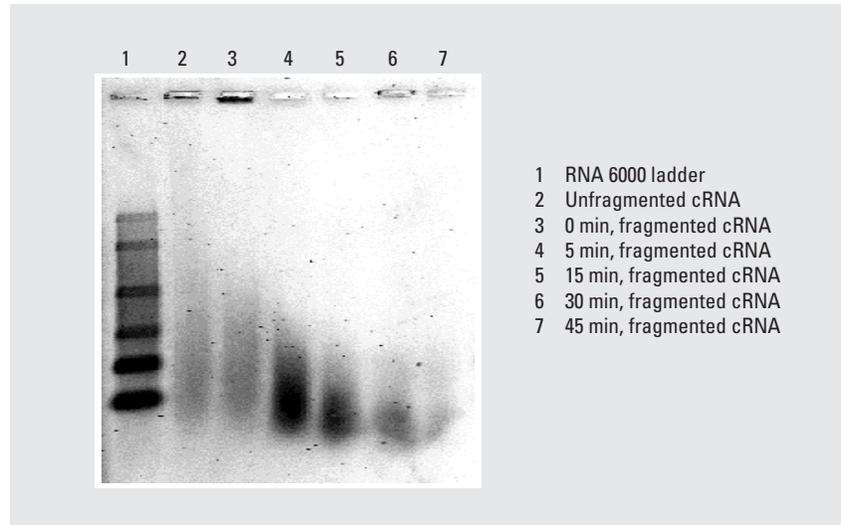


Figure 5
1 % agarose gel stained with SYBR Gold nucleic acid stain. The gel was run at 100V for 1.5 hours. The first lane contains the RNA 6000 Ladder from Ambion Inc., with fragment sizes of 200, 500, 1000, 2000, 4000, and 6000 bases. Lanes 2-7 contain the HeLa cRNA fragmentation time course samples in the following time point order: 0, 5, 15, 30, and 45 minutes. The gel was scanned with a Fluorimager™ 595.

the cRNA fragments in the sample will be in the 50–200 base pair size range. This amount of detail can not be observed by running the same samples on an agarose gel (figure 5). The gel provides a rough estimate of the fragment size, but is less informative about the extent of fragment distribution and concentration. By running a fragmentation time course using the Agilent 2100 bioanalyzer, it is possible to optimize cRNA fragmentation reactions and identify how long a reaction needs to proceed so that a majority of the sample is concentrated within the desired size range for optimized target specificity.

4. Hybridization to an oligo array

The final procedure in the gene expression workflow is the hybridization of the fragmented Cy3 and Cy5 labeled cRNA samples to an oligo microarray followed by data extraction and analysis. Genes expressed in equal levels will appear yellow in a pseudo-color image of the array, while differentially expressed genes will appear as shades of either red or green. The brightness of each spot reflects the hybridization specificity, as well as the relative abundance of that gene in the two labeled populations (figure 6). The Cy3 and Cy5 cRNA samples generated for this experiment were created from the same HeLa poly A+ mRNA template. For

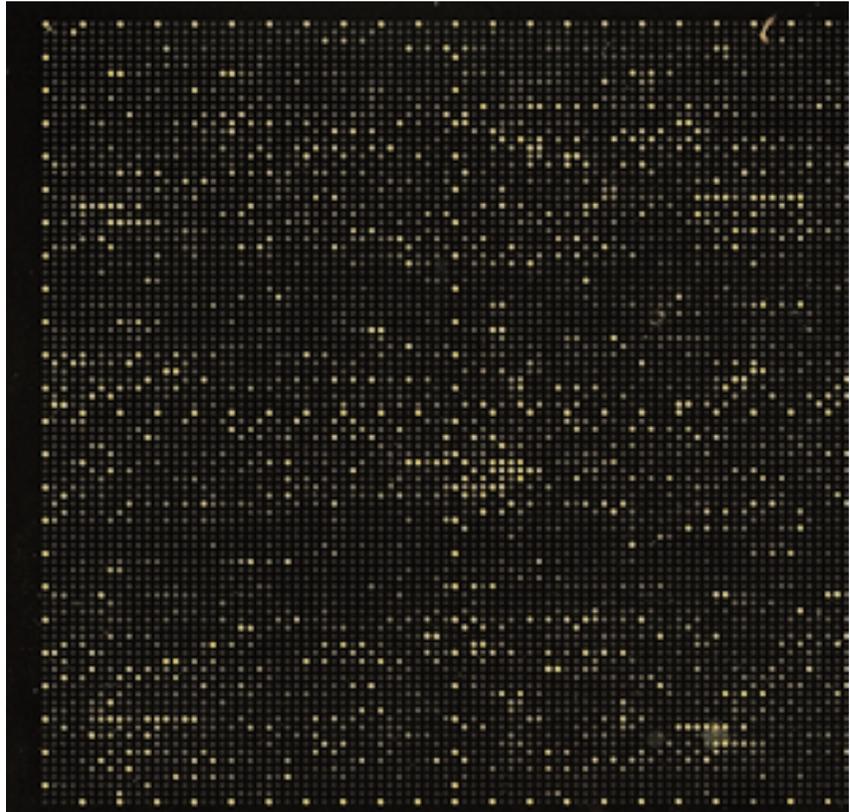


Figure 6
Image from a cRNA array hybridized with fluorescent targets from identical Cy5 (red) and Cy3 (green) labeled HeLa samples.* Genes expressed in equal levels will appear yellow, while differentially expressed or labeled genes will appear as shades of either red or green. The brightness of each spot reflects the relative abundance of that gene in the two labeled populations. The microarray was scanned with a confocal dual-laser scanner at 532 and 633 nm (Agilent Technologies).

** In print, picture is a black and white representation of a color gene array image. To see original color scheme, view our electronic version at <http://whadmin.pal.agilent.com/WHRoot/00022685/00026403.pdf>*

each of the 3000 unique features on the array, the Cy3 target intensity and Cy5 target intensity were expected to be equivalent.

One way to represent microarray data is in an intensity plot (figure 7) in which each array feature is plotted with its Cy3 log intensity on the y axis and its Cy5 log intensity on the x axis. When the two intensities associated with a feature are identical, the data point will lie along the diagonal. Genes that are differentially expressed will lie above or below the diago-

nal. On the graph, lines indicating a two-fold and three-fold difference in Cy3/Cy5 log intensity have been drawn to clearly identify the differentially expressed features. Consistent with fluorescently labeled targets generated from the same HeLa mRNA source, a majority of the array features in figure 7 fall along the diagonal, indicating that the labeling and fragmentation reactions were equivalent for both fluorescently labeled targets. Many of the features had intensities greater than 1000 (Log 3.0) indicating an abun-

dance of statistically relevant target produced in the cRNA amplification, as well as good fragmentation resulting in successful hybridization. The bioanalyzer electropherograms of the amplified labeled cRNA and fragmented cRNA enabled the prediction that the targets would perform well on the array prior to hybridization.

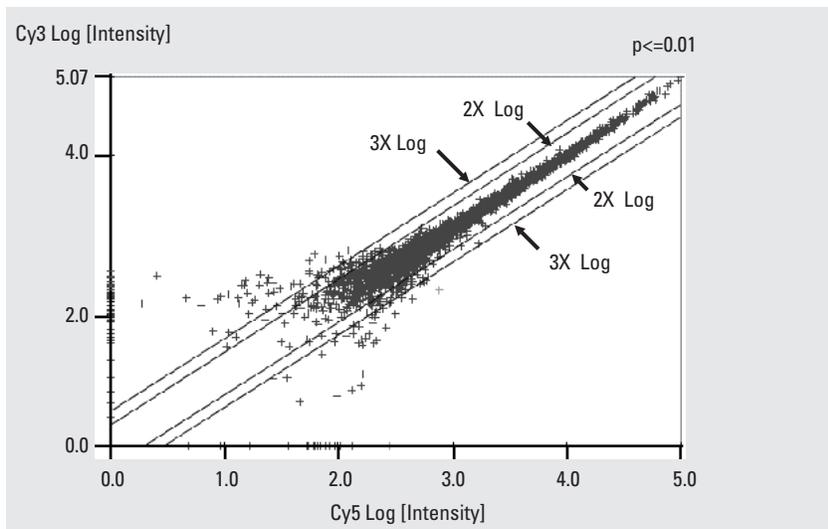


Figure 7
Log intensity plot of Cy3 HeLa cRNA vs. Cy5 HeLa cRNA. In the data analysis, the positive and negative control points were removed, so that only the 3000 unique features were being analyzed. This plot shows all of the data points that fall within a 99 % confidence level. The higher the intensity of the feature, the more statistically relevant it is. In our graph, nearly all points with an intensity above 1000, log(3), fall along the diagonal within the 2X Log [intensity] lines, indicating that the performance of the array, hybridized with our fragmented cRNA samples was very good.

Conclusions

Using the Agilent 2100 bioanalyzer and the RNA 6000 LabChip kit, the generation of fragmented cRNA samples can easily be monitored through visual inspection of the electropherogram at each step along the sample workflow. The bioanalyzer can be used to qualify the integrity of RNA templates, check the success of cRNA amplification reactions and ensure sufficient sample fragmentation. These quality control assessments at key points in the sample preparation will increase your confidence that further oligonucleotide microarray experiments will perform well.

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