# Advancing the quality control methodology to assess isolated total RNA and generated fragmented cRNA

Application

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**Abstract**

This Application Note describes how Gene Logic, Inc., has improved its ability to analyze the quality of total RNA and fragmented cRNA by using the Agilent 2100 bioanalyzer. The Eukaryote Total RNA Nano and Pico assays are currently being utilized to examine the 28S/18S ratio in the total RNA as well as detection of possible degradation. The mRNA Smear Nano assay is used to confirm that a high percentage of the cRNA sample is fragmented to lengths in the optimal range necessary to be hybridized onto Affymetrix microarrays.

Agilent Technologies

## Introduction

Gene Logic Inc. is a leading provider of integrated genomics information and bioinformatics products and services related to gene activity in human disease and toxicity that enable global pharmaceutical and biotechnology companies to optimize the time, risk and cost of drug discovery and drug development. In order to do this, the quality control methods must ensure that only the highest quality samples proceed through our high-throughput process, which in turn help to reduce the downstream costs of cDNA synthesis, In-Vitro Transcription and, finally, hybridization onto microarrays. For qualitative purposes, many laboratories continue to analyze their RNA samples using MOPS gels with gel electrophoresis. However, an internet search of Microarray facilities around the country shows an increase in the usage of the Agilent bioanalyzer.

When using gel electrophoresis to examine the quality of samples, pre-cast MOPS gels were used. Every now and then a problem with the gels was encountered, either user related or gel related. Some general problems occurring were sample migration, mold spots in the pre-cast gels and staining difficulties. The key issue associated with the RNA quality control gel was that at times the 18S and/or 28S bands of the total RNA were not clearly visible on the gels making the interpretation difficult. When running fragmented cRNA samples on MOPS gels it was often difficult to distinguish whether or not the samples were allowed a sufficient amount of time to fragment because of poor resolution. Problems with MOPS gels would lead to re-running of the samples on new gels, therefore wasting valuable time and money. The samples that should have failed would be caught by quality control measures later on in the process, but would ultimately cost the company money on reagents in the steps leading up to that.

The Agilent 2100 bioanalyzer combined with the RNA 6000 Nano LabChip Kit provides key advantages to running MOPS gels. The Agilent bioanalyzer data is easy to interpret and the chip runs on the bioanalyzer are less time consuming. It can take up to an hour and half to prepare the samples, run a gel, stain the gel, and take a picture. The whole Agilent process (sample preparation to saving the file) can be done in half that time. There is no improper staining and no shortened or lengthened run times. Also, because each chip has the capacity to run 12 samples, at approximately 30 minutes per chip, this makes the Agilent 2100 bioanalyzer a useful tool for high throughput screenings, especially with multiple bioanalyzers.

## Results

### Need for a new assay

The goal was to find a new assay to be used to evaluate the quality of the total RNA and fragmented cRNA. This was needed because there was occasional inconsistency with the use of MOPS gels. The qualitative analyses should be as definitive and dependable as possible. Problems associated with MOPS gels in a high throughput environment may be due to user error. Common mistakes seen in the laboratory are: accidentally piercing the gel while loading, not loading enough of sample into the well, improperly securing the gel to the gel rig, not running the gel on the appropriate voltage/time or not staining the gel properly. These errors are reflected in the gel images below (Figures 1-6). These problems would be eliminated using the Agilent 2100 bioanalyzer.

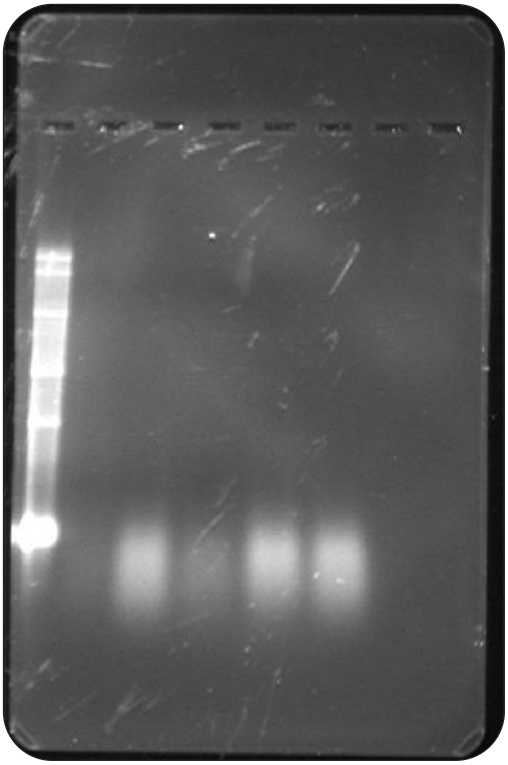

**Figure 1**
**This fragmented cRNA gel was placed crooked in the gel rig. The ladder almost ran off the side of the gel. The ladder is also not of good quality (possibly overloaded).**

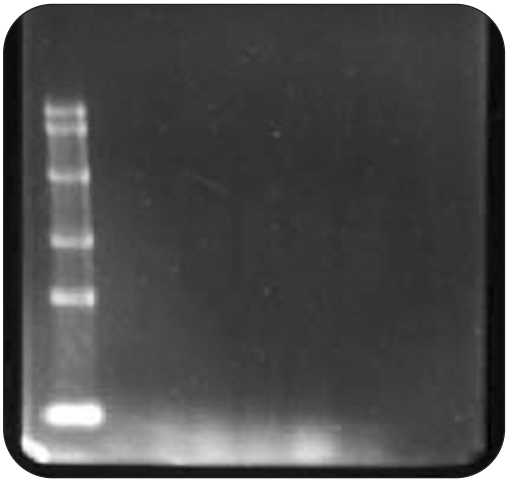

**Figure 2**
**This fragmented cRNA gel ran for too long. The samples have almost completely run off the gel.**

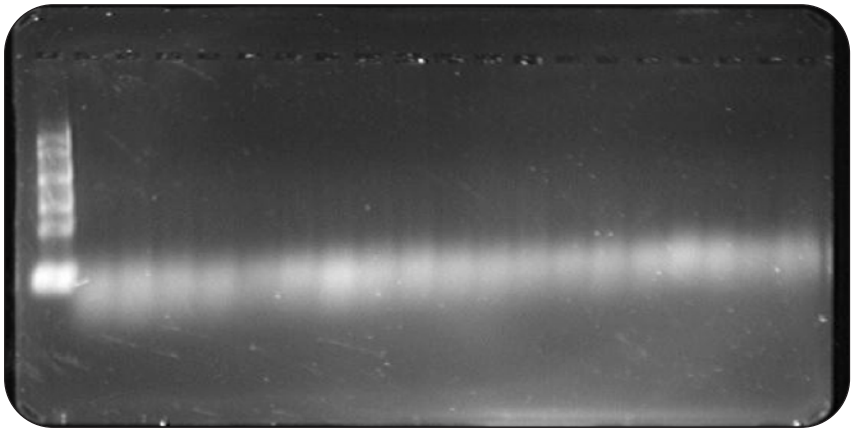

**Figure 3**
**It appears that in this gel the voltage spread was uneven. The migration of the samples is not balanced.**

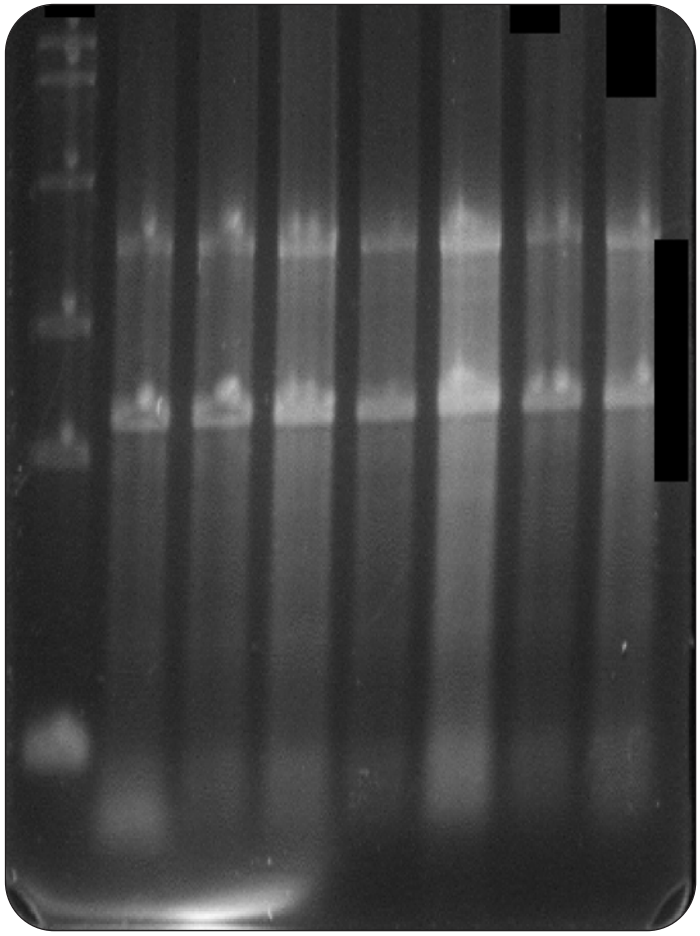

**Figure 4**
**In this total RNA gel, all of the lanes seem to have been pierced during loading of the samples.**

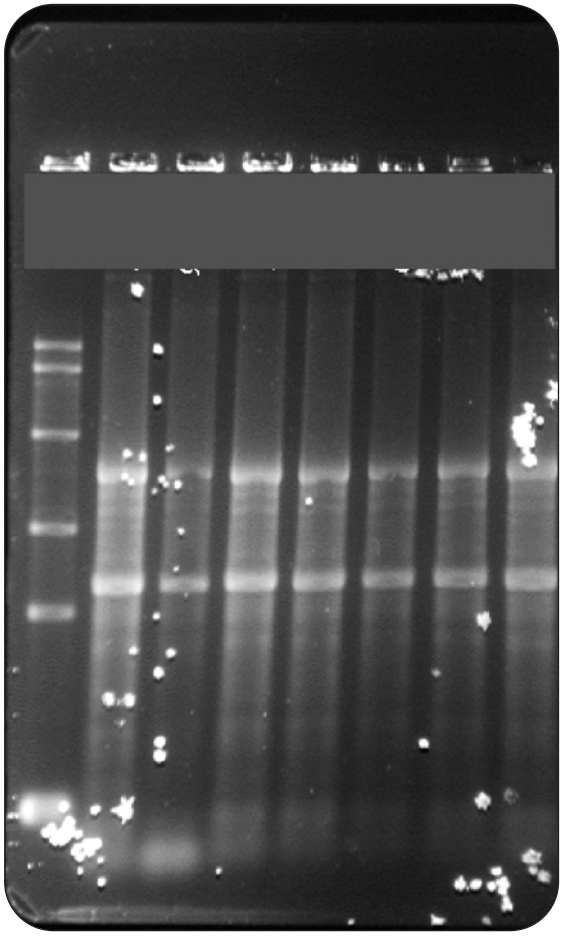

**Figure 5**
**This gel has some type of mold growing in it that is fluorescing with the stain.**

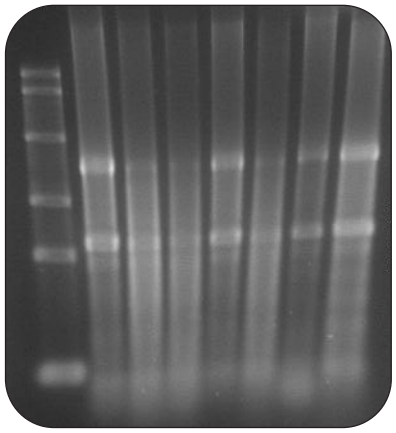

**Figure 6**
**This gel has merging lanes due to problems with the electrodes.**

MOPS gels may be suitable to get an idea of the quality of samples, but the gels do not always clearly reveal a detailed image of the sample. It is important that the genomics information GeneLogic, Inc. provides to its customers is of the highest quality. Therefore, there needs to be an accurate, reliable way of assessing the quality of the samples.

### Agilent 2100 bioanalyzer vs MOPS gels for Total RNA

The Agilent 2100 bioanalyzer was tested as a tool for quality control for total RNA and compared against the MOPS gels for the same samples over a series of weeks. During this time a grading system was derived for the sample data using the Eukaryote Total RNA Nano assay with the bioanalyzer. It was determined that the samples were either given a "pass", "fail", or "Pass – needs additional QC" rating. "Pass-Needs additional QC" would essentially

mean that the sample was passed, but that it would from then on be closely monitored for its quality in further steps. Additional Quality Control assays would be employed to further evaluate the quality of these samples. For the MOPS gels a system was already in place to either pass or fail a sample. Because of the clarity of the electropherograms produced using the bioanalyzer, distinguishing between the high quality samples and poor quality samples became easier. For example, when samples were analyzed using both the bioanalyzer and the MOPS gels, some of them that were failed using the Agilent software were passed using gel electrophoresis, and vice versa. Some samples that were passed using the bioanalyzer and failed on the MOPS gel are shown in Figures 7 and 8. Based upon the electropherograms, the acceptable quality of the samples is clearly determined, yet on the MOPS gel images a ribosomal band is missing from each.

Just as the MOPS gel is capable of giving false negatives, it is also capable of giving false positives. Some examples of samples that were passed on the MOPS gel but failed on the bioanalyzer are shown in Figures 9 and 10. (Note: Samples that were passed using the MOPS gels were failed by QC measures further along in the process)

Based upon these observations, there are clearly more distinguishable results on the electropherograms produced

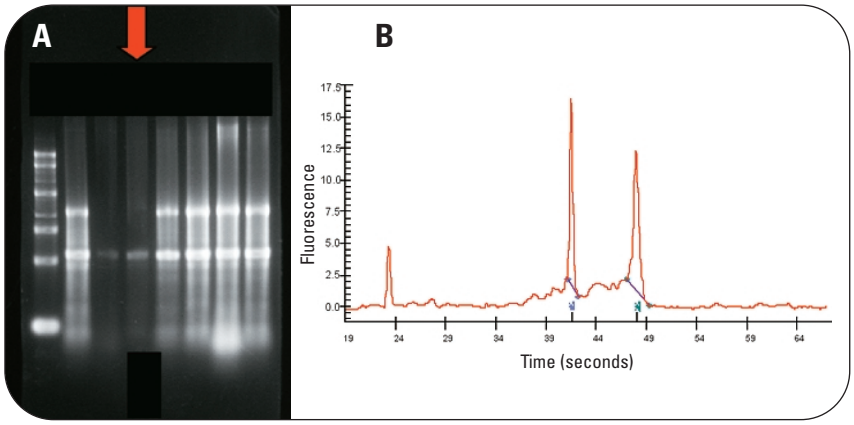


**Figure 7**
**Part a) shows the band absence in the lane marked with an arrow, yet the two bands are clearly seen as peaks in the electropherogram in b). The 28S/18S ratio for this sample is 1.09.**

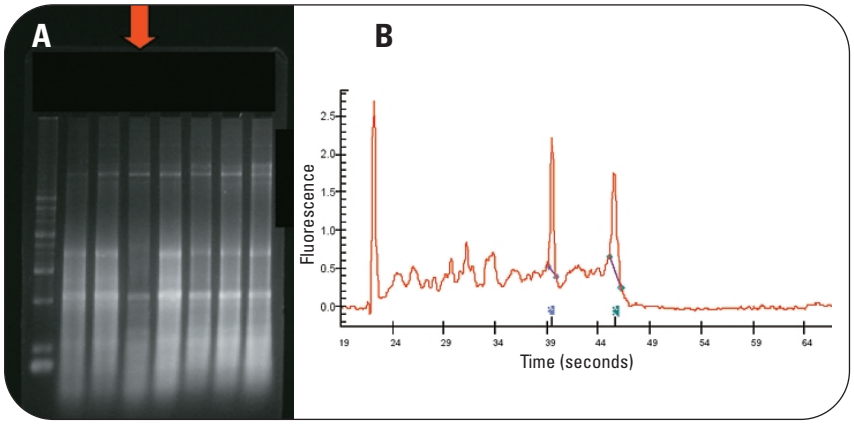


**Figure 8**
**Part a) marked with an arrow shows a missing ribosomal band, while Part b) shows both the 18S and 28S bands present (note that some degradation is present, therefore additional Quality Control measures may be needed). The 28S/18S ratio for this sample is 0.90.**

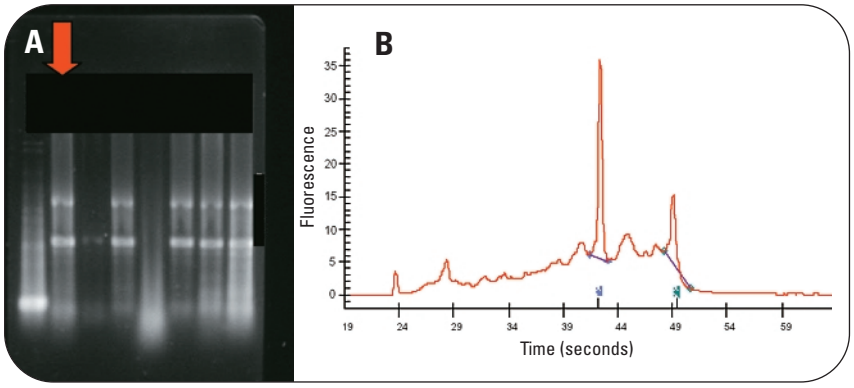


**Figure 9**
**Part a) marked with an arrow shows the 18S and 28S bands as both present (also note the bad quality of the ladder). Part b) shows a greatly diminished 28S peak. The 28S/18S ratio for this sample is 0.43.**

using the bioanalyzer then there are in the MOPS gel images. There is a lot more room for human and mechanical error using the MOPS gels, which may contribute to the apparent differences.

### Agilent 2100 bioanalyzer vs MOPS gels for fragmented cRNA

When analyzing fragmented cRNA, what is examined is that the sample has been allowed to heat long enough and is fully fragmented to a desirable level. The size of the fragments can be estimated using the ladder on the MOPS gel, but this is not conclusive and determination of fragmentation is very subjective due to the poor resolution of low molecular weight components (refer to Figures 1-3). The mRNA Smear Nano assay used with the bioanalyzer is capable of showing the desired fragment length range and percentage of the total area for each sample. This is an extremely valuable tool because it takes the subjectivity away from the determinations. The software allows the user to isolate the range of fragment lengths necessary for proper hybridization onto microarrays, and gives the user the percent of the total area that the range covers. These tools are utilized to ensure that the samples have been fully fragmented to the proper size range. If the samples do not have a high enough percentage of total area in the proper size range then they are heated for an additional amount of time to allow adequate fragmentation. Examples of varying levels of fragmentation are shown below (Figures 11-15).

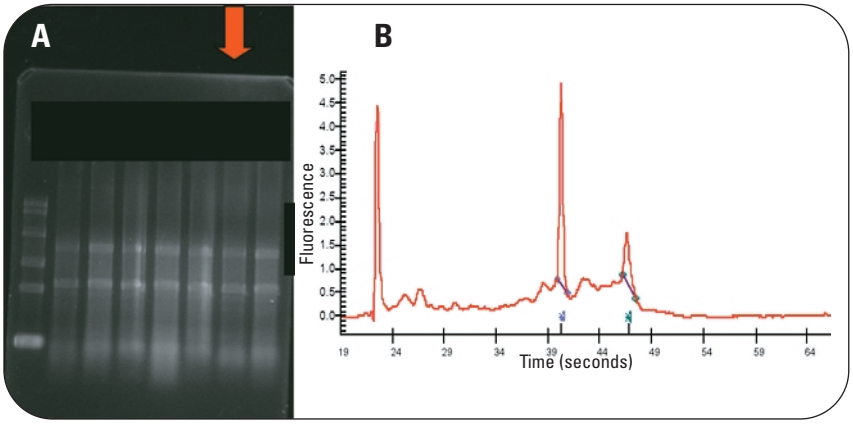


**Figure 10**
**Part a) marked with an arrow shows the two ribosomal bands present, albeit weak (possibly due to poor staining). Part b) shows a 28S peak that is too small, and therefore warrants failing. The 28S/18S ratio for this sample is 0.35.**

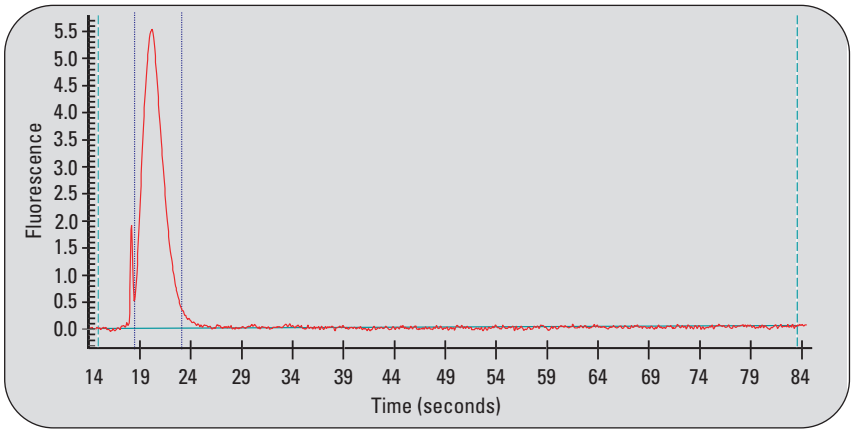


**Figure 11**
**The blue dotted lines outline the target area of fragmentation. The desired fragment lengths make up 95.32% of the total area of sample detected.**

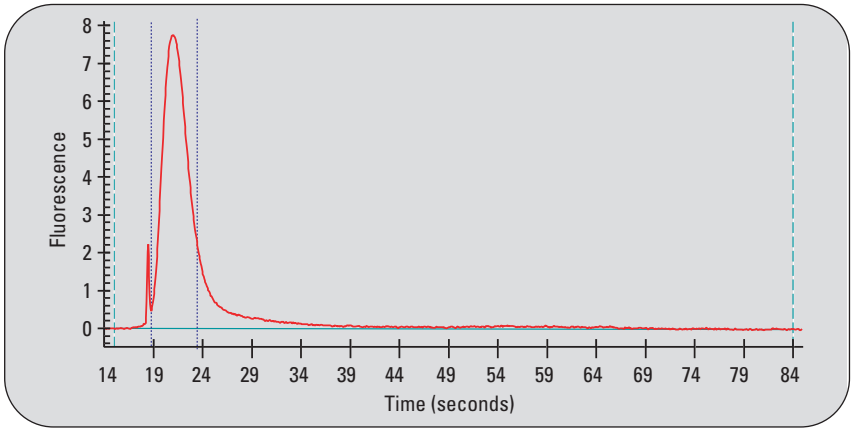


**Figure 12**
**In this electropherogram a short tail is starting to form on the end of the sample. This portion of the sample has not fragmented to the preferred lengths, but there is still enough sample in the optimal range for hybridization. The percentage of total area for this detected sample is 81.63%.**

The mRNA Smear Nano assay is clearly a useful means for examining the fragmented cRNA samples. In a MOPS gel image it is not always easy to tell whether or not a sample is fragmented to sufficient lengths necessary for hybridization. Samples that may need to be heated longer may not be recognized, and their hybridization onto the arrays may be affected. By using the Agilent 2100 bioanalyzer it is easy to see which samples are not fully fragmented.

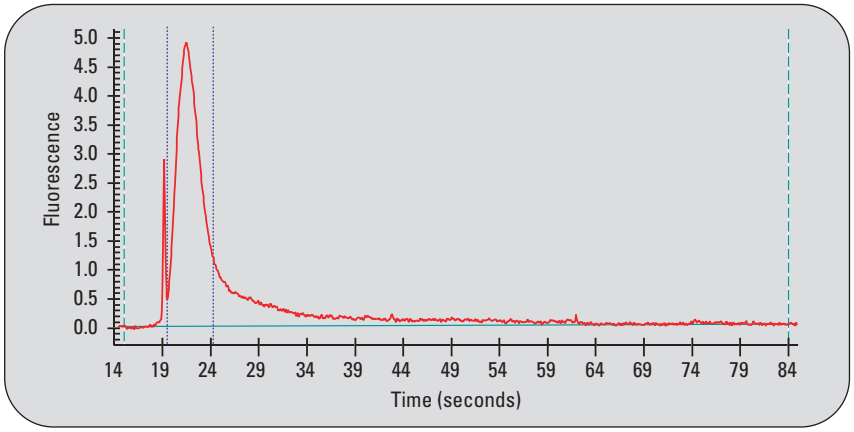


**Figure 13**
**A larger tail is forming in this sample indicating that this sample may need to be heated for a longer period of time. The percentage of the total area for this detected sample is 73.41%.**

## Conclusion

The Agilent 2100 bioanalyzer and the RNA 6000 LabChip Kit were used to analyze the quality of the total RNA and fragmented cRNA. The Eukaryote Total RNA Nano and mRNA Smear Nano assay gave clear, distinct results. It is easy to draw conclusions from the electropherograms created using the Agilent software. The whole Agilent process is less time consuming, less subjective, more reliable, and is suitable for use in a high throughput environment. Money could also be saved on reagents, microarrays and samples because more concrete data is being generated with the Agilent 2100 bioanalyzer. Use of the Agilent 2100 bioanalyzer also helps ensure Gene Logic, Inc.'s customers that the data they are providing to them is of superior quality.

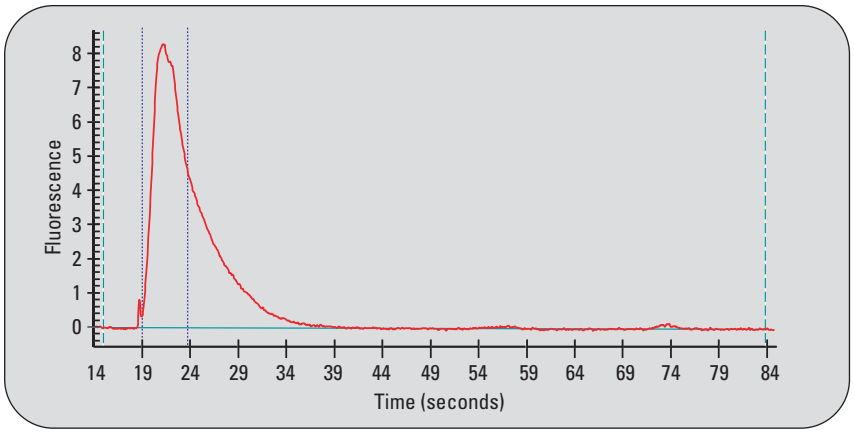


**Figure 14**
**This sample has obviously not been heated long enough. There is a massive tail of underfragmented sample. The percentage of total area for this detected sample is 65.58%.**

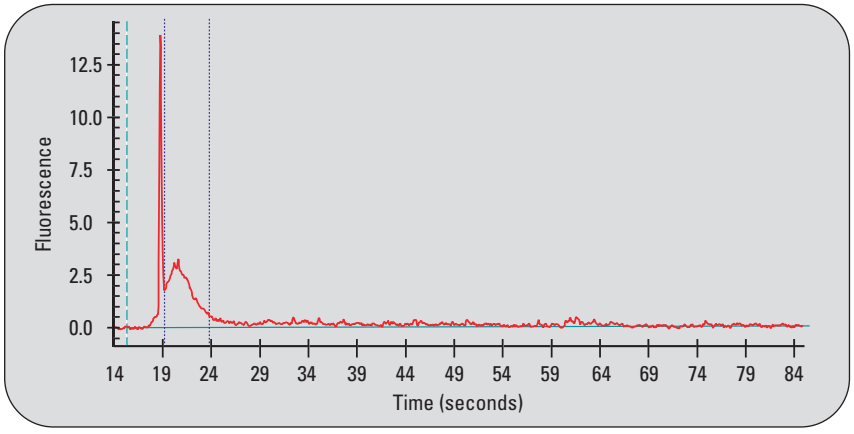


**Figure 15**
**This sample was overfragmented. The sample has degraded into small fragments, most of which are not detected. The percentage of total area for this detected sample is 65.21%.**

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Published July 1, 2003
Publication Number 5988-9861EN

**Agilent Technologies**