Novel technique for analyzing RNA from the actively growing culture of *Escherichia coli* using Agilent 2100 Bioanalyzer

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Molecular characterization of pathogens (bacteria, fungal, protozoa, and virus) and other microorganisms is very critical for proper diagnosis and drug development. In this study, actively growing Escherichia coli culture was used for automated analysis of variability of the culture. Normally the pathological analysis is done through microscopic visual examination, which is the traditional way of study. This is not however, a precise methodology because it has some variability depending on a variety of factors. A more specific method of analysis is needed in order to evaluate the variability and to allow for a more precise characterization of the types of pathogens that are the causal organisms of a particular disease. A novel technique coupled with fluorescence can eliminate some variability in pathogen sampling and both techniques can further enable the study of pathogens. Significant development was achieved in advancement of equipment through refinement of technology in the last decade and there has been an enormous effort to understand the molecular mechanisms of diseases and the way that drugs interact with molecular targets. At the very beginning the focus was on the research of sequencing the human genome and identifying genes associated with specific diseases, the novel technique arrived later for identifying the pathogens by using the knowledge of the science of microfluidics. The Lab on a Chip technology utilizes microfluidics, where sample preparation, fluid handling, and biochemical analysis are performed on a microchip. Data processes are analytical and fully automated eliminating the variability seen with other methods. The Lab on a Chip technology utilizes samples of various sizes of RNA, proteins, and DNA profiles to characterize the pathogen samples collected depending upon which chip is used. Fluorescent microscopy shows the detailed similarities and dissimilarities in morphology including the size and shape. These similarities and dissimilarities are needed for characterizing the pathogens and establishing the comparative accounts among them. These techniques can eliminate the variability and errors in sampling the pathogens and facilitate a quick and efficient collection of diagnostic data in pathology of various samples.

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Introduction

The science of molecular pathology, which encompasses the molecular characterization of pathogens and other microorganisms through the atmosphere, is integral to our understanding of pathogen dispersal mechanisms and is a valuable tool for disease forecasting [1, 2]. Much of the plant pathology literature on aerial spore dispersal is based on spore samples collected by the Burkard 7-day volumetric sampler and its progenitor, the Hirst 24-h sampler [2, 3]. Earlier, immune-blotting and binding assay were done to characterize Amb a 1, a major allergen from Ragweed pollen [4]. Over the last decade there has been an enormous effort to understand the molecular mechanisms of diseases and the way that drugs interact with molecular targets [5]. The RNA 6000 Pico LabChip® kit uses very small amounts

of RNA for the quality control utilizing the Agilent 2100 bioanalyzer. The main advantages of the RNA 6000 Pico LabChip kit are its high sensitivity combined with its time saving experimental procedure [4]. Here, we show experimental data that demonstrate the sensitivity of the RNA 6000 Pico LabChip kit by the analysis of different amounts of highly diluted RNA samples. The results show the suitability of the RNA 6000 Pico LabChip kit for quality control of total RNA and messenger RNA (mRNA) in a range from 200 pg/µl to 5000 pg/µl and 500 pg/µl to 5000 pg/µl, respectively. The reproducibility of the assay is also demonstrated [6]. Agarose gel electrophoresis followed by ethidium bromide staining can be used to check the purity and quality of an mRNA preparation. However, for accurate analysis, this traditional technology typically requires significant amounts of sample and an additional method for quantification [7]. RNA concentration is typically determined by measuring the absorbance at 260 nm in a spectrophotometer. The ratio between the absorbance at 260 and 280 nm gives a rough estimate of RNA purity with respect to contaminants that absorb at a different wavelength, such as proteins, but provides no information regarding neither possible ribosomal RNA contamination nor potential degradation by nucleases during the purification process [8]. With the increased interest in expression profiling applications, there has been a steadily growing demand for faster, more automated analysis tools that consume minimal sample amounts. Lab-on-a-Chip technology is particularly suited for the rapid analysis of nucleic acids because it integrates multiple experimental steps [1-5]. The purpose of this study was to evaluate and demonstrate a new method to assess the variation among potential pathogens.

Materials and methods

Actively growing culture of *Escherichia coli* was obtained using LB media after two days of incubation at 37^{o}C. 200 µl aliquot tube of Elution Solution was heated in water bath at 70-80°C. 300 µl of *E. coli* in LB medium was obtained. The cells in culture were centrifuged at low speed centrifugation (3,000 rpm/min). The supernatant was removed thoroughly. Once the culture medium has been removed the cells were kept on ice at all times. 300 µl of Lysis/Binding solution was added to the pallet and was vortexed for 30 sec to 1 min. The cells were pipetted up or and down vigorously with a micropipette. The detailed requirements are listed under the Appendix section of this paper.

Steps to make the lysate/ethanol solution:
350 µl of 100% ethanol and 350 µl of Lysis/Binding solution were added into an eppendorf tube. The mixture was mixed thoroughly by pipetting up and down vigorously.

Lysate/ethanol solution mix was added to a spin filter inside an eppendorf tube. The tube was centrifuged for about 1 min at 10,000 rpm. The flow was discarded in a biohazard container and collection tube and the filter were reused. 300 µl of wash solution #1 was added to the filter and was centrifuged for about 1 min at 4,000 rpm. The flow was discarded in a biohazard container and collection tube was reused. The tube was centrifuged for another 1 min at 4,000 rpm. 2.5 µl of DNAse I and 17.5 µl of DNase I Buffer were added to the filter which was placed in a collection tube. The tube was centrifuged for about 1 min at 4,000 rpm and the flow was discarded. The collection tube was reused. 700 µl of wash solution #1 was added to the filter to wash and it was centrifuged for 1 min at 4,000 rpm and the flow was discarded. The collection tube was reused. 500 µl of wash solution #2/3 was added to the filter to wash and it was centrifuged for 1 min at 14,000 rpm and the flow was discarded. Again the collection tube was reused. The last 2 steps were repeated and the tube was centrifuged for an additional 2 min. 40 µl of pre-heated elution solution was added to the center of the filter that was kept in the collection tube. The tube was centrifuged for 30 sec at 14,000 rpm. 20µl

of pre-heated elution solution was again added to the center of the filter that was in the collection tube and was centrifuged for about 30 sec at 14,000 rpm. 60µl of pre-heated elution solution was added to center of the filter that was kept in the collection tube. Contents were centrifuged at 14,000 rpm for 30 sec. The reagents were allowed to equilibrate at the room temperature. 550 µl of RNA 6000 Nano gel matrix were placed into a spin filter and centrifuged for 10 min at 15, 000 rpm and the flow through was discarded after into a biohazard container. 65 µl of filtered gel were placed into 0.5 ml RNase-free microfuge tubes. The RNA 6000 Nano dye concentrate was then vortexed for 10 sec then centrifuged for about 1 min at 4,000 rpm. 1 µl of dye was added to a 65 µl aliquot of filtered gel. After usage, the tube was capped and vortexed thoroughly. A new RNA Nanochip was removed from the sealed bag and placed on a chip priming station. 9.0 µl of gel dye mix was inserted into the chip well by pipette where it was made sure that no bubbles were added when placed into the well (Figure 1: A and B). It is important that the plunger is set at 1 ml and the chip priming station is closed firmly. The lock latch would click when the Chip Priming Station is closed properly. The plunger was pressed down gently until it was held by the syringe clip. Precisely 30 sec were allowed to pass before the plunger was released with the clip release mechanism. Another five seconds were allowed to pass where the plunger was pulled back slowly to the 1 ml position. The Chip Priming Station was opened so that 9.0 µl of gel-dye mix was pipetted into each of the wells marked “G”. 5 µl of the RNA 6000 Nano Marker was placed into the well marked with the ladder symbol and into each of the 12 wells. The ladder was denatured for two minutes in a 70°C water bath before use. 1 µl of each sample was put into wells marked with the ladder symbol. Samples were denatured (70°C, 2 min) before 1 µl of each sample was loaded into each of the 12 sample wells on the chip. The chip was vortexed for 1 min at 2,400 rpm where any liquid spilled on the top of the chip was carefully removed with a clean sterilized tissue paper. The chip was placed into the Bioanalyzer, lid was shut properly and the appropriate software and programs were run to obtain the results.

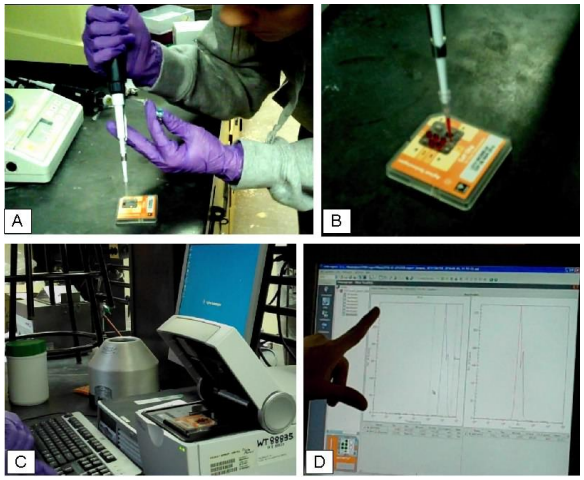

Figure 1. A-D showing the steps of working with a Lab on a Chip and analyzing data. **A.** Loading the sample to the Chip. **B.** A chip loaded with sample. **C.** The Chip placed into the Bioanalyzer. **D.** Computer screen showing the Electropherogram.

Results and discussion

After two days of inoculation, the *E. coli* subculture developed a mild milky color. Throughout the course of filtering and centrifuging, it should be noted that the solution that sat on top of the filter before centrifugation must appear at the bottom of the collection tube after it was centrifuged. If this was not the case, further spinning via centrifugation should be done until the liquid sets at the bottom of the collection tube. It was noticed that volume of the liquid (often on the nanogram scale) was extremely small. Pipetting small amounts (1-9 µl) of liquid into the chip should be done very carefully to avoid errors. If not done cautiously it might result into false negatives. As the ladder was being analyzed in the Agilent 2100 Bioanalyzer, the screen displayed many red peaks that would later be used for sample analysis. If these peaks are not

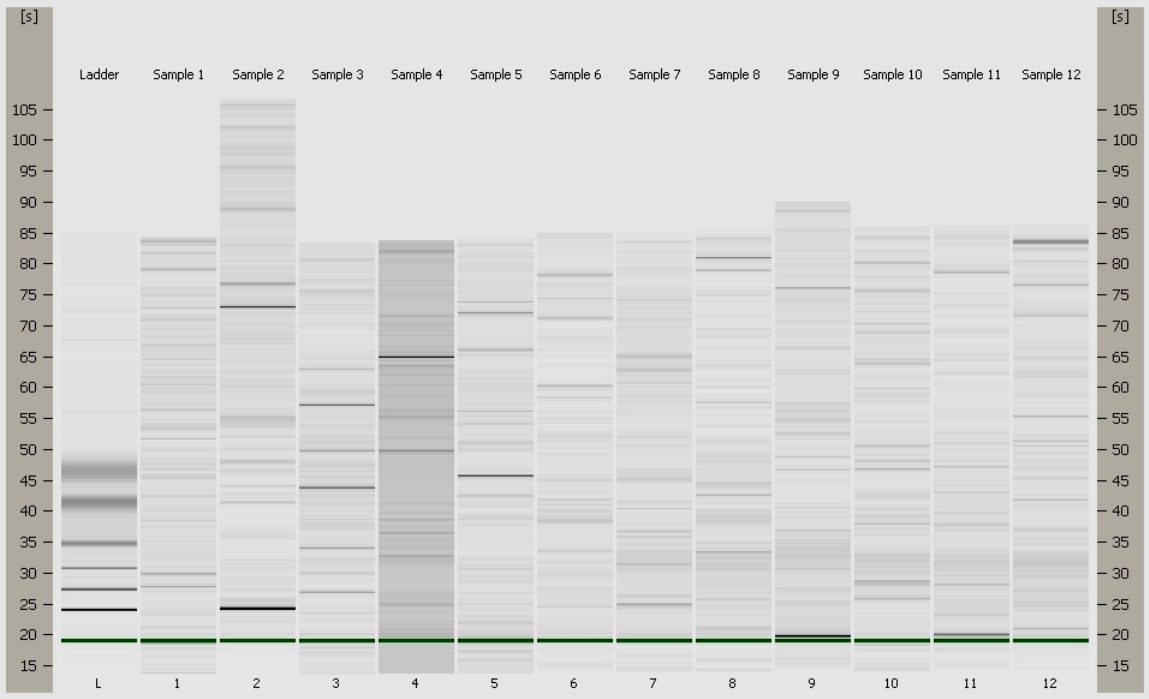

Figure 2. Showing the Gel figure of ladder (left, labeled "L") and 12 samples (labeled 1-12).

noticed, troubleshooting should be considered for the remainder of the samples. As samples were being analyzed, most of them were displaying peaks around 20 sec along the x axis followed by other peaks that were characteristic for each sample. The results were then displayed via an Electropherogram which is a graph that shows time versus fluorescence (Figure 3). Also, a gel like figure was also acquired from the analysis of the ladder and each individual sample.

Quantitation of nucleic acids is essential in many research procedures in molecular biology. However, accurate determination of DNA or RNA concentrations can be difficult, particularly when the purity of the sample is uncertain. In many cases, the most rapid and accurate approach for determining the concentration of relatively pure nucleic acids and proteins is absorbance spectrophotometry [8, 9].

The Agilent 2100 Bioanalyzer provides a platform that employs a fluorescent assay involving electrophoretic separation to evaluate RNA samples qualitatively. This Lab-on-Chip technology is a much needed technology in biotechnology that needs to be thoroughly researched for its broad range of uses today. The technology has many advantages in that it utilizes a very small amount of sample (µl) versus the many milliliters of sample that must be used in a conventional flow cytometer. The fact that the Lab-on-Chip has the ability to analyze many samples within a brief period is definitely an advantageous feature that is not readily available in other flow cytometer techniques. The Agilent 2100 Bioanalyzer is a small bench-top system that is no bigger than a desktop computer. In our experiment, the *E. coli* culture was grown up very actively so that a concentrated amount of RNA could be used for analysis. Centrifugation was employed to either concentrate a solution from its solutes or to separate the solute from the solvent. The lysis binding solution was used to lyse or break open the cells so that the genetic information inside the cell could be released from the cellular

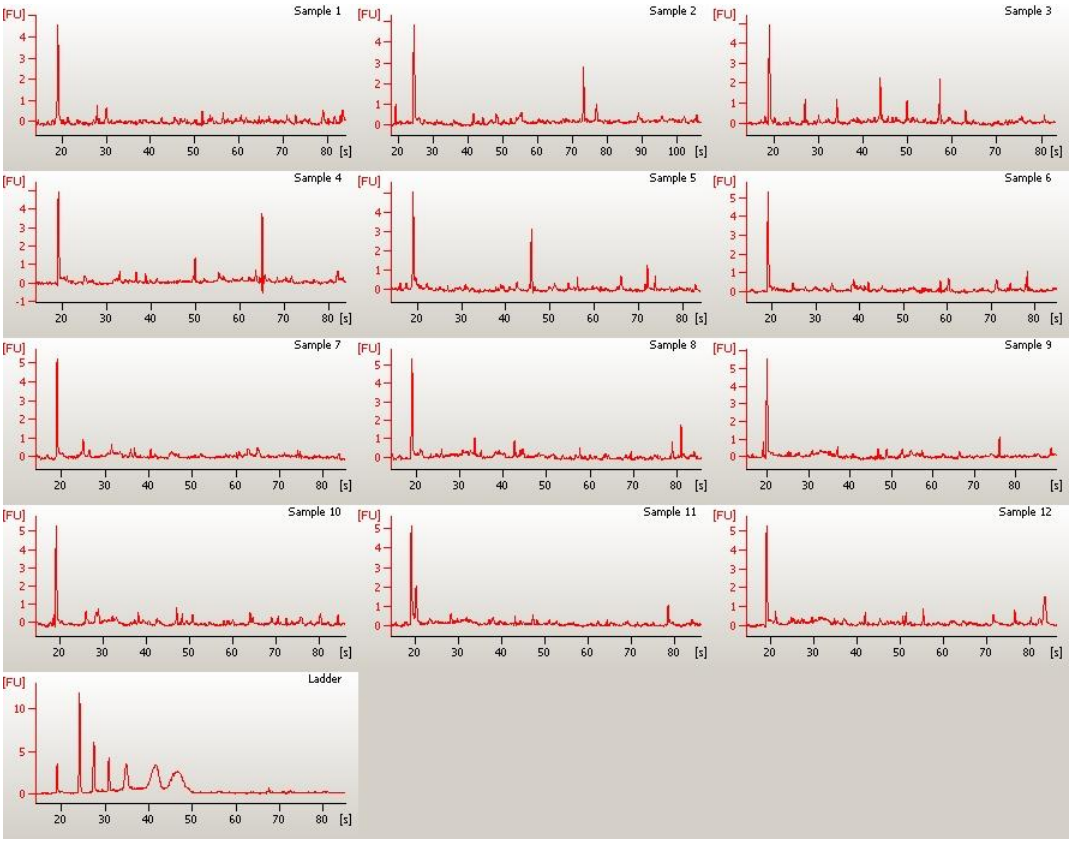

Figure 3. Showing the Electropherogram of Ladder and 12 samples.

compartments or organelles where it is usually housed. Once the lysate was added, the RNA had to be separated from this solution. At this point 100% ethanol and lysis/binding solution were added for separation of the two entities. A DNase treatment was used to remove any genomic DNA that could possibly contaminate the RNA that was attempted to be isolated. This step is usually used when PCR cannot or does not distinguish between products amplified from DNA and RNA templates. An elution step must be accomplished after the previous step and is ready to be analyzed at this point. After the ladder and other reagents were pipetted onto the micro-chip (Figure 1: A and B) analysis was performed on the samples.

It is known that the Agilent 2100 Bioanalyzer (Figure 1: C) measures the amount of fluorescence as the RNA sample is pulsed through a micro-channel over a period of time. An electropherogram is created by the software which diagrams fluorescence over time (Figure 1: D). Smaller molecules are pulsed through the separation channel quicker than the larger molecules and will appear on the left side of the Electropherogram. A good Electropherogram (Figure 3) will have well defined peaks (20s) with low noise ratio which are the smaller peaks usually due to low molecular weight species. It can be concluded that the majority of the samples had low molecular weights since their large fluorescent peak were seen around 20s. The analytical graphs should have two distinct peaks, one that is a marker peak and another broad hump which is characteristic of intact RNA. In some samples RNA may have been degraded exhibiting the spectrum shifts toward earlier migration times. However, since there was one prominent peak, we concluded that RNA was in fact in every one of the twelve samples analyzed. The gel (Figure 2) that

accompanies the Electropherogram (Figure 3, sample 1-12) is a visual representation that is comparable to an agarose gel used in conventional DNA and RNA analysis.

Conventional agarose gels can consume a large amount of sample and can require other steps in the quantification of molecules. In this experiment, the ladder marker seen at around 18/19 on the gel is seen on all other twelve wells. The other marks on the gel allow us to recognize that other molecules with larger weights were also in the sample.

This technology can also be employed for the analysis of DNA and protein assays. This kit is designed to check the quality and determine the concentration of RNA samples through the use of a low marker. This allows for the sample alignment and comparison of samples based on electrophoretic traces. In the mid 90s the validity of nucleic acid purities was monitored by absorbance ratios [9]. In the beginning of the previous decade, Microfabrication technology was used to develop a system consisting of disposable glass chips containing etched channels, reagents including polymer matrix and size standards, computer-controlled instrumentation for performing electrophoretic separations and fluorescence detection of double-stranded DNA, and software for automated data analysis [10].

The outcome of gene expression experiments is strongly influenced by the quality of RNA starting material. The quality of RNA depends on the purity of the tissue from which it is isolated. This fact is important in cancer research, where local gene expression can change between neighboring cell populations. Some new tools allow the isolation of individual cells out of heterogeneous populations [5]. Until now, good techniques for the analysis of RNA in the picogram range weren't available. A new analysis kit based on Lab-on-a-Chip technology is now closing this gap. It works on the same principle as the nanogram range kits that have become standard procedure for RNA quality control [6, 9]. In conclusion, the RNA 6000 LabChip kit was employed successfully using the Agilent 2100 Bioanalyzer to analyze various RNA samples prepared in our laboratory. Twelve samples were separated on the LabChip through a single separation channel. The chip run was complete in 30 minutes and data for each sample was assessed in real time after each separation. Preparation, analysis and clean up all were done in a brief period with significant data output that can further be analyzed as needed.

Conclusions

Proper characterization of the pathogens is very critical for accuracy in diagnosis and development of vaccines and therapeutics. The older methods followed long and tedious processes that sometimes resulted in erroneous data. The novel technique demonstrated in this study appears to be an efficient method consisting of improved and fast techniques to assess the variations among potential pathogens. With further refinement in technology to reach accuracy in data production the Lab-on-Chip technology may prove to be very useful in quick and efficient characterization of pathogens that is of immediate need for fast detection and treatment of various diseases.

Appendix

Requirements:

1. Actively growing culture of *Escherichia coli*
2. LB media (800 ml deionized water, 10 g Bacto-tryptone, 5 g yeast extract, 10 g Sodium Chloride, pH 7.5)
3. Agilent 2100 Bioanalyzer
4. Water bath
5. Centrifuge
6. Ethanol (100%)
7. Lysis/binding solution:
   Lysis Solution was prepared by adding 500 µL of β-mercaptoethanol (β-ME) to the

bottle containing Lysis Solution (Agilent Total RNA Isolation Mini Kit, Product Number 5185-6000). To prepare smaller amounts, 10 µL of β-ME is added per 1 ml of Lysis Solution. The Lysis Solution was stored at 4 °C after the addition of β-ME.

8. Vortex machine
9. Gel Cell culture medium
10. Lysate
11. Wash solution #1
12. Spin Filters (2)
13. Eppendorf Tubes (10)
14. DNase I
15. DNase I Buffer
16. Wash solution #2/3
17. Elution solution
18. RNA 6000 Nano Chip
19. RNA 6000 Nano Reagents and Supplies

a. RNA Nano Dye Concentrate
b. RNA 6000 Nano Marker
c. RNA 6000 Nano Gel Matrix

20. RNA 6000 Ladder
21. Gloves
22. Goggles
23. Micropipette and compatible tips (1000 µl, 200 µl and 20 µl)
24. Autoclaved Deionized water

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