



**THE LABYRINTH**  
**Indiana State Department of Health Laboratories Newsletter**



**Indiana State  
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 Laboratories**

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**Our Mission:**

*The Indiana State Department of Health Laboratories partners with other public health agencies to provide timely and accurate information needed for surveillance and outbreak investigations to protect and improve Hoosier health.*

**Public Health will never be the same, thanks to  
 Whole Genome Sequencing**

**By Jamie Yeadon-Fagbohun**

CDC’s PulseNet program celebrated its 20<sup>th</sup> anniversary in 2016. PulseNet USA is a nationwide collaboration of laboratories that connect foodborne illness cases to detect outbreaks. An estimated 270,000 illnesses are prevented every year since PulseNet was implemented.<sup>1</sup> There are 83 different laboratories throughout the United States with at least one PulseNet laboratory in every state.<sup>2</sup>

Pulse-field gel electrophoresis (PFGE) is used to make a DNA fingerprint of bacteria which is then imaged and loaded into a local database and uploaded to the PulseNet national database. The DNA fingerprint is made by cutting the DNA using specific enzymes and running them through an agarose gel that is infused with varying electrical currents. DNA moves away from negative electrical currents. The larger the piece of DNA, the slower that migration occurs, and the closer the resultant band is to the loading well.<sup>3</sup> The results, or PFGE pattern, can be seen in Figure 1. PFGE is completed for foodborne pathogens such as *Salmonella*, *E. coli*, *Listeria*, *Shigella*, *Campylobacter*, and *Vibrio*. A cluster is defined as an increase in the local database of a specific PFGE pattern that is twice the expected number of matching patterns for that area during a specified time period. Nearly 1500 clusters are identified by state and local agencies along with 30 multistate outbreaks each year.<sup>4</sup> PFGE has long been the “gold-standard” for outbreak detection of foodborne pathogens.

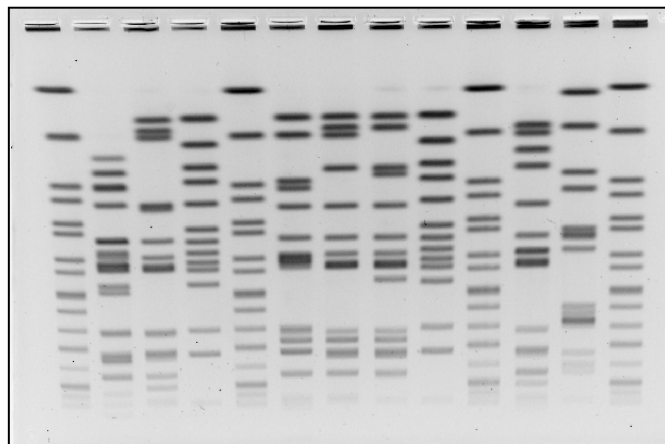


Figure 1 (*Salmonella* PFGE)

In 2014, the CDC’s Advanced Molecular Detection (AMD) initiative started. This began the process of implementing Whole Genome Sequencing (WGS) on a national scale, which will allow public health labs to identify, characterize, and complete outbreak detection from a single test. States were awarded money to purchase instruments and implement the process in the CDC’s Epidemiology Laboratory Cooperative Agreement (ELC) grant. By the end of 2017 the goal is to have at least one lab per state running WGS for *Salmonella*, *E. coli*, and *Listeria* in real-time.<sup>5</sup>

The Indiana State Department of Health Laboratories (ISDHL) brought on WGS technology in 2016 with the purchase of two Illumina MiSeq instruments. They’ve been

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“Public Health will never be the same, thanks to Whole Genome Sequencing” (continued from page 1)

colloquially named Sheldon and Leonard (after the characters from the television show ‘The Big Bang Theory’). We currently have three staff members certified in WGS for PulseNet organisms. Between June 2016 and February 2017, we completed sequencing on 250 specimens: 11 *Listeria* isolates, 89 *E. coli* isolates, and 150 *Salmonella* isolates. We are still working on optimizing our workflow to sequence all of these pathogens in real-time. We are also in the process of training and certifying additional staff members for WGS.

WGS consists of 5 distinct steps: DNA extraction, library preparation, cluster generations, sequencing, and data analysis. It takes about 2 days to culture and extract the specimens for WGS. It takes another 1-2 days for library preparation and to have the specimens ready for WGS. Once the specimens are loaded on the instrument, it takes about 2 days for it to complete the run. DNA extraction is done from a pure colony patch. Library prep is done to obtain nucleic acid fragments with adaptors attached on both ends as well as with indices attached. This is done through two PCR reactions and a bead-based cleanup of the DNA. Cluster generation and sequencing are completed using the Illumina MiSeq instrument. Approximately 16 specimens can be tested on each run of the instrument. Cluster generation occurs on the instrument flow cell which allows for thousands of copies of the DNA to be created and bounded to the flow cell for the sequencing. See Figure 2 for Illumina’s diagrams for Library Preparation and Cluster Generation.<sup>6</sup>

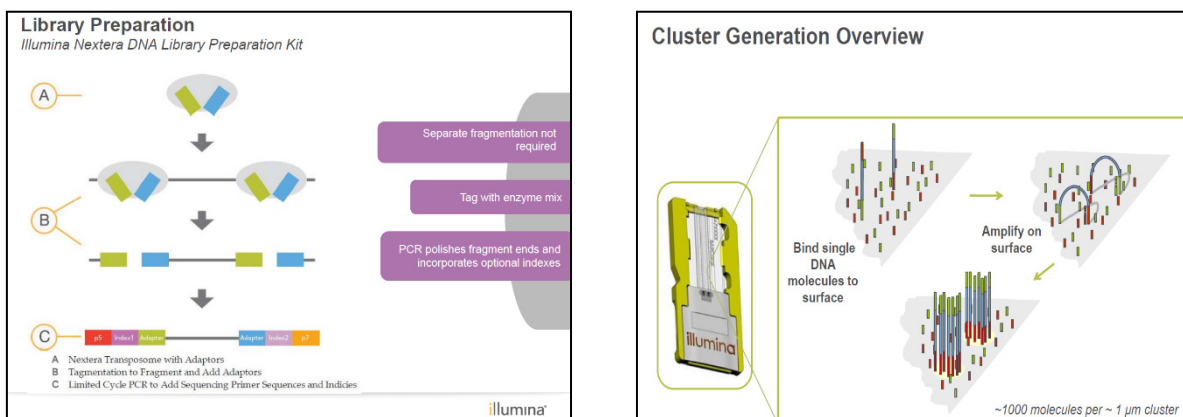


Figure 2 (from Illumina PDFs of Presentations):

Once the clusters have been generated, the instrument begins sequencing. This protocol calls for paired-end sequencing by synthesis. This means that the sequencing is completed by taking advantage of the DNA reaction to the addition of polymerase. The flow cell is then imaged and because the sequencing contains fluorescently labeled nucleotides, an image is created for each base. See Figure 3 for Illumina’s diagrams for sequencing and imaging.<sup>7</sup>

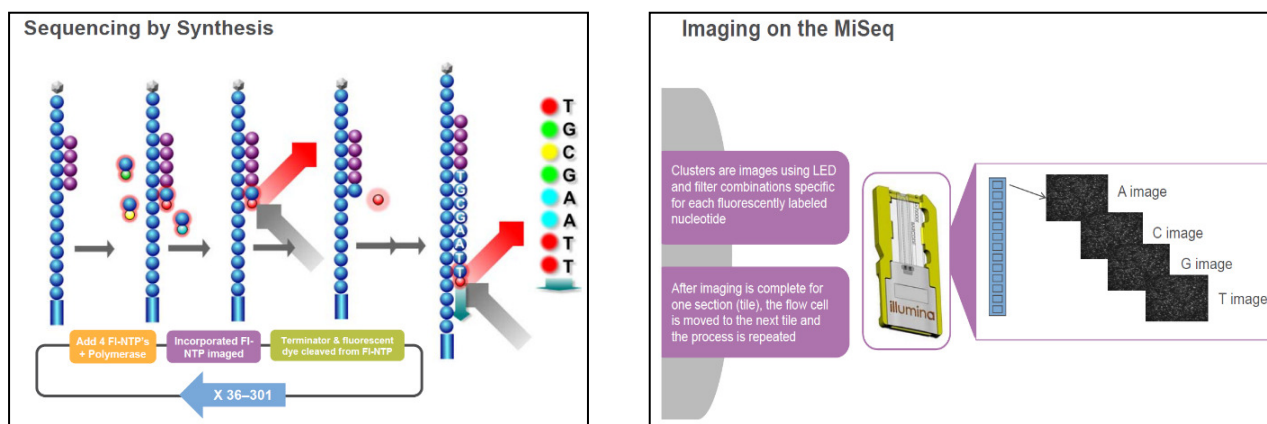


Figure 3 (from Illumina PDFs of Presentations):

Once the instrument run is complete, it is time for analysis. Before beginning analysis, the basic QC of the run is reviewed to ensure that there is quality data. This includes looking at the Q-score, cluster density, clusters passing filter, and estimated yield (Figure 4). The rest of the data analysis is not currently completed in-house. Quality runs are shared with CDC PulseNet and NCBI so that the analysis can be completed.<sup>8</sup>

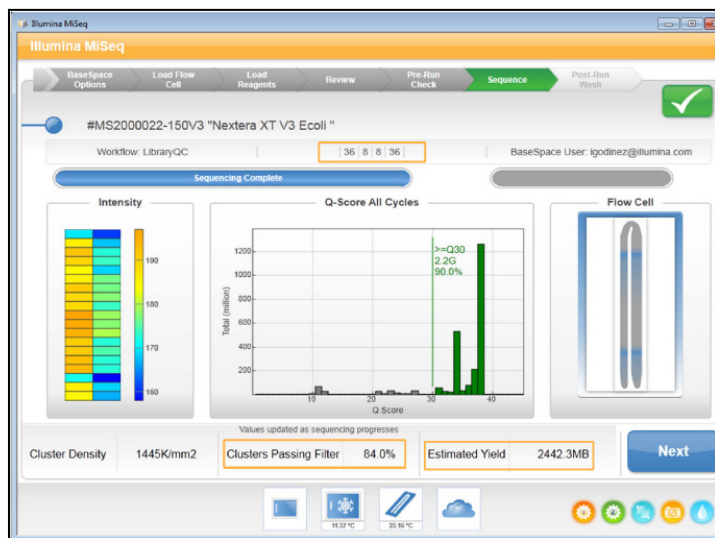


Figure 4 (from Illumina PDFs of Presentations):

At this time, PulseNet is only certifying labs for the laboratory portion of this WGS analysis but we anticipate that they will start providing analysis certification this summer. We are looking forward to being able to complete some of the data analysis in-house. We anticipate that the data analysis will be similar to what occurs with PFGE, where you are looking for relatedness of specimens. Figure 5 shows a phylogenetic tree of some *Salmonella* Reading isolates in the national database. PNUSAS003096 is an Indiana isolate that has been included in this outbreak. It shows that there is very little genetic difference between the specimens with the blue bracket and a large genetic difference between those in the blue bracket and the additional specimen at the top. Overall, ISDHL is excited for this new technology and type of data analysis, and the tremendous impact it will have on public health response to address foodborne pathogen outbreaks!

**References:**

- <sup>1</sup> <https://www.cdc.gov/pulsenet/anniversary/timeline.html>
- <sup>2</sup> <https://www.cdc.gov/pulsenet/about/fast-facts.html>
- <sup>3</sup> <https://www.cdc.gov/pulsenet/pathogens/pfge.html>
- <sup>4</sup> [https://www.cdc.gov/pulsenet/pdf/pulsenet-20-years\\_4\\_pg\\_final\\_508.pdf](https://www.cdc.gov/pulsenet/pdf/pulsenet-20-years_4_pg_final_508.pdf)
- <sup>5</sup> <https://www.cdc.gov/pulsenet/anniversary/timeline.html>
- <sup>6</sup> R:\LABS\Molecular\Sequencing\NGS\Illumina\PDFs of Presentations
- <sup>7</sup> R:\LABS\Molecular\Sequencing\NGS\Illumina\PDFs of Presentations
- <sup>8</sup> R:\LABS\Molecular\Sequencing\NGS\Illumina\PDFs of Presentations

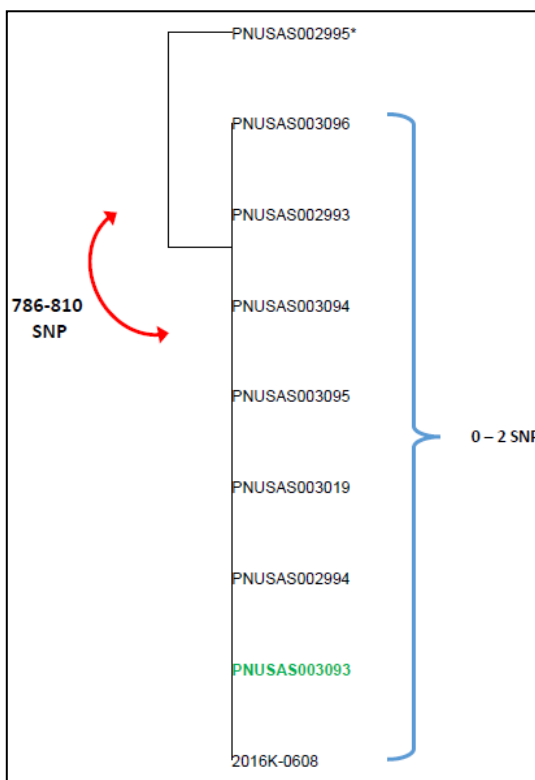


Figure 5 (from CDC Join Sharepoint Site for PulseNet)

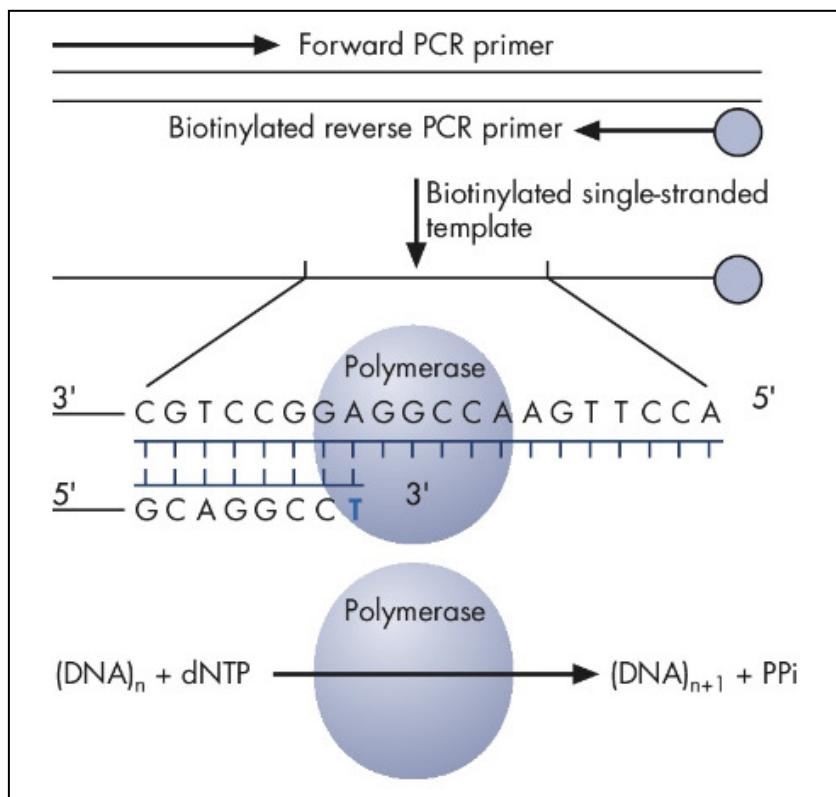
## Rapid Detection of Multiple Drug Resistant Tuberculosis (MDR TB)

By Jessica Gentry

The Indiana State Department of Health (ISDH) TB Laboratory utilizes the technique of pyrosequencing to rapidly detect antibiotic resistance to *Mycobacterium tuberculosis* complex (MTBC). In contrast to traditional culture-based antimicrobial susceptibility testing, which requires a pure culture and can take several weeks, pyrosequencing can be performed directly on processed sputum specimens and can be completed within a few hours.

Pyrosequencing quickly and accurately obtains short sequence data from targeted genes of MTBC. The sequences generated are compared to sequences of library strains with known antibiotic resistance patterns. This process requires several steps. First, MTBC DNA is amplified using specific biotinylated-primers spanning the bacterial genome, similar to PCR. Next, the amplified gene fragments are sequenced with primers specific to the region containing the possible mutations. Like in PCR, nucleotides are incorporated stepwise; however, in the case of pyrosequencing, the incorporated nucleotides are added one at a time (ex. A, then G, then C, then T) instead of all at once. Incorporation of a given nucleotide at each step causes the generation of ATP, which reacts with a luciferin substrate and produces a phosphoretic/luminescent signal that can be detected by the pyrosequencing instrument. If a nucleotide is not incorporated, or if there are additional nucleotides remaining after the reaction, the enzyme apyrase degrades them in order to prevent false signals. Correlating the identity of the added nucleotide and the presence of a phosphoretic signal gives us the sequence. The sequencing portion of this procedure is performed on the Qiagen PyroMark Q24 Pyrosequencer.

The most critical application of this technology in the ISDH TB Laboratory is to identify multiple drug resistant TB (MDR TB), which is TB that is resistant to a minimum of Rifampin (RIF) and Isoniazid (INH). TB infections are typically treated with four antibiotics simultaneously, two of which are RIF and INH. Mutations at the gene *rpoB* account for approximately 90% of RIF resistant strains, and mutations at *katG* and *inhA* account for over 90% of INH resistant strains. The ISDH TB Lab routinely performs pyrosequencing testing for all three of these loci for all newly identified TB patients.



Step 1 - A DNA segment is amplified and the template strand is biotinylated. The primer and single stranded template are incubated with enzymes and substrate.<sup>1</sup>

Figure 2: Step 3 - ATP sulfurylase converts PPI to ATP, which drives the conversion of luciferin to oxyluciferin that generates light.<sup>1</sup>

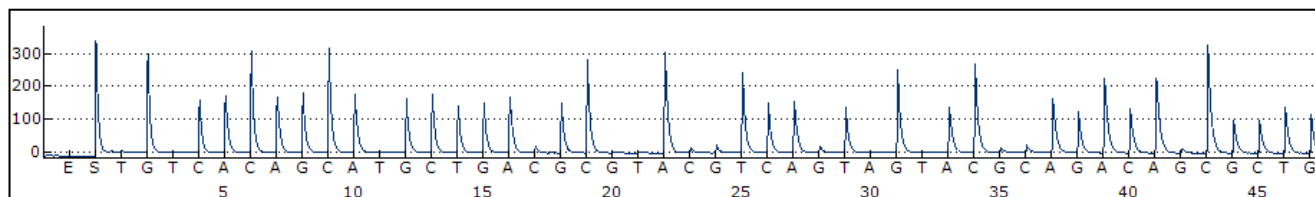
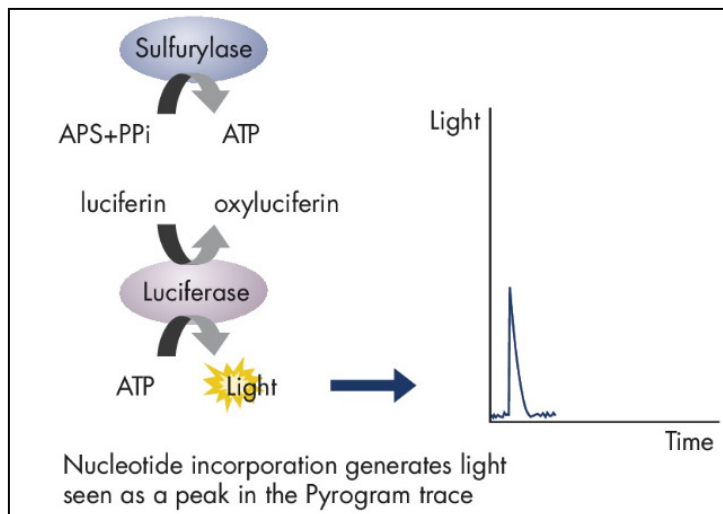


Figure 3: Pyrogram of wild type *rpoB* (from ISDH Lab data)

Although MDR TB is relatively rare in Indiana, pyrosequencing has been used to rapidly detect several cases of single drug resistance in recent years, and was most recently used to detect an especially challenging MDR-TB case. This case was from a symptomatic patient at a local health department. The patient had never been tested for TB before, but possessed many of the common risk factors and classic symptoms for active TB disease, including cough, fever, night sweats, and weight loss. The sputum was collected and arrived at the ISDH lab the next day. After being identified as a TB case by PCR on the following day, pyrosequencing testing was initiated. Less than a week later, mutations were detected for both *rpoB* and *katG*. The mutations indicated that the patient was highly likely to be resistant against both RIF and INH, making this a case of MDR TB. The sample was immediately forwarded to the CDC, where confirmatory testing revealed additional resistances to several more antibiotics, including Ethambutol, Pyrazinamide, Streptomycin, Kanamycin, Amikacin, and partial resistance to Capreomycin and Ethionamide. The patient was only one antibiotic away from being classified as Extensively Drug Resistant (XDR TB). Using traditional methods, it would have taken many more weeks to provide this diagnosis!

In this situation, pyrosequencing results allowed TB Control to quickly realize the severity of this patient's TB strain and to adjust the therapy accordingly. Not only was the patient's outcome greatly improved, but other patient contacts were tested rapidly. Several of these contacts had latent TB, which would typically be treated with INH. Pyrosequencing results allowed TB Control to employ more effective therapies for these patients, thus preventing the onset of active TB disease. Due to the rapid action and close collaboration of the laboratory and TB Control, there were no cases of transmission from this patient. Pyrosequencing played a key role in this story, which is an excellent example of public health infrastructure working optimally.

#### Sources

1. Qiagen website—[www.qiagen.com](http://www.qiagen.com)
2. CDC MDDR manual—[www.cdc.gov/tb](http://www.cdc.gov/tb)

## I Ate What?!

By Mary Hagerman



Shrimp and rice dish

Have you had a delicious shrimp and rice dish lately? Shellfish, rice, and its cooking water may all contain arsenic. If it does, and you eat it, your urine can contain arsenic. Should you be worried?

Maybe your well water contains high amounts of the metal, Chromium. The well may be contaminated from industrial waste containing hexavalent chromium (Cr6+), or it may contain a form of chromium that is an essential nutrient (Cr3+). How can you tell?

Those are the questions that a Liquid Chromatograph – Inductively Coupled Plasma/Mass Spectrometer (LC-ICP/MS) answers. An LC-ICP/MS analyzes different kinds (species) of Arsenic, Chromium, Mercury and several other metals. Each species may or may not be harmful; each displays a different toxicity. The Arsenic content found in shellfish usually is an organic compound and is relatively harmless; the Arsenic in rice or water usually is an inorganic form, specifically the arsenic species of III (As 3+) or V (As 5+), both of which are

toxic, with As 3+ being about 60 times more toxic of the two. Unfortunately, well water contaminated by Arsenic is common in Indiana due to geological factors. To remove the arsenic coming from wells, water conditioning companies will often develop treatment protocols based on the type of Arsenic present, because they need different removal techniques for each species.

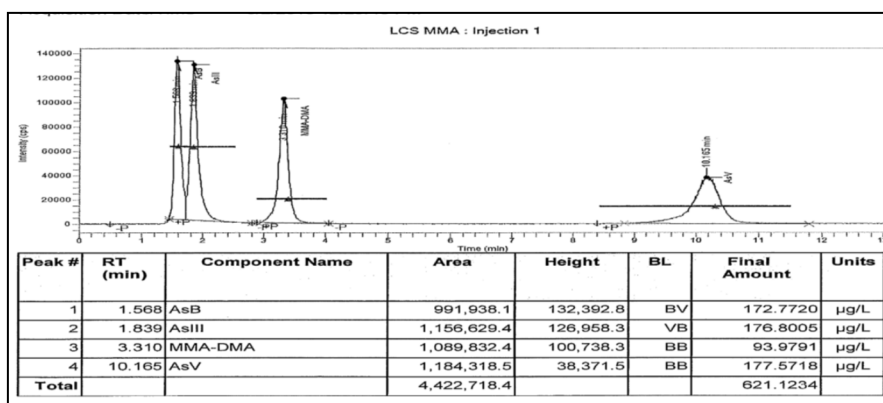
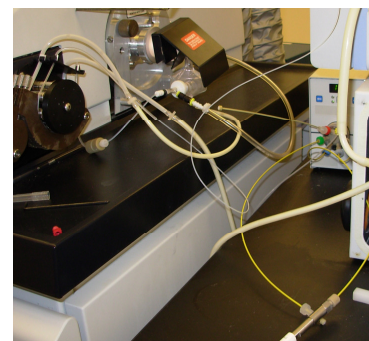


Figure 1 (Image courtesy of Taylor Dao)

In Figure 1, each peak represents a different species of Arsenic. All types of samples, (rice, fish, and even urine) can be analyzed on an LC-ICP/MS system. It starts with a prepared sample flowing through a liquid chromatography column that separates each metal species by size and charge. As the liquid exits the column, each species is detected by using 6000 to 10,000 degree Celsius argon plasma, which quickly dries and separates the liquid into individual atoms and ions. The charged ions are directed into the mass spectrometer which permits those ions at each mass to be counted. For Arsenic, we are looking at mass 75, a number directly from the Periodic Table. For the Chromium species we usually look at mass 52, because it is the most abundant isotope mass. The number of ions at each mass is proportional to its concentration so that the ICP/MS can determine the total concentration of a metal at any time. Without the LC separation, though, we could not calculate how much of each species is present.

At ISDH we sometimes need to find out the form of a metal. Does a poorly labeled fish paste contain Arsenobetaine, a relatively non-toxic Arsenic compound? Or, does it contain Methylmercury, a very toxic organometallic compound? Does a non-regulated, traditional medicine product for infants contain toxic amounts of Arsenic 3+? The use of the LC-ICP/MS is common for both the ISDH Food laboratory and the Clinical Chemistry laboratory where we check food, personal care products, and urine for different metal species. Thanks to funding from both the FDA and CDC Preparedness funds, we are able to answer these questions.



Right: Liquid Chromatograph–Inductively Coupled Plasma/Mass Spectrometer (LC-ICP/MS)

## Real-Time PCR in the ISDH Laboratory

By Erica Vecchio, MS

Since its discovery by Kleppe and Khorana in the late 1960s<sup>1</sup>, the use of polymerase chain reaction (PCR) has revolutionized the modern diagnostic laboratory. PCR now serves as one of the primary methods for diagnosing and managing numerous infectious diseases<sup>2</sup> and detecting emergent conditions, such as new strains of established pathogens<sup>3</sup>. In public health laboratories, which are expected to rapidly and accurately respond to health issues and possible outbreaks, PCR forms the foundation of molecular diagnostic procedures. In the ISDH Laboratory nearly every department, from the Food and Dairy area to Biowatch, utilizes PCR at some point during the testing process.

In conventional PCR, a specific DNA (or RNA) sequence is amplified primarily for the purpose of creating multiple copies of a tiny portion of DNA or in order to compare separate samples for abundance. This is accomplished through a sequence of events. First, the DNA sequence of interest is heated so the strands of DNA are separated (denaturation). Next, primers are added and allowed to bind to the end of one strand and the other end of the second strand through cooling (annealing). Thermostable polymerase enzymes begin to synthesize complementary single-stranded DNA sequences at the primer points of each strand. Thus, the DNA strands are replicated in opposite directions (elongation). The entire process is repeated numerous times, usually around 40 or so cycles, until exponential numbers of the DNA sequence are produced. In the final step, the amplified DNA mixture is cooled and may be stained in an agarose gel for visualization; the brighter the dyed band, the more DNA copies produced<sup>1</sup>. When PCR is performed on RNA, the target RNA must first be converted to DNA before amplification.

Recent advances in technology have improved the efficacy of PCR. At the ISDH Laboratory, QuantStudio™ DX Real-Time PCR Instruments will soon be introduced into a few departments, including Assay Development, Virology, and Tuberculosis. Although real-time PCR operates under the same principles as conventional PCR, there are some significant advantages. Through the addition of fluorescent dyes, PCR product is monitored and measured in real-time during each cycle by the machine's instrumentation. Moreover, the ability to accurately measure the amount of amplicon (number of PCR product molecules) allows the analyst to precisely quantify the amount of initial material in samples. Measurements are made during the exponential phase of the PCR reaction, which is the optimal point for data analysis. Due to the accuracy of the real-time PCR process, use of a gel or other post-procedure techniques are unnecessary since analysis is automatically done during the assay process. Finally, only a single tube is used during the assay, thereby decreasing possible contamination and analyst effort<sup>4</sup>.

The use of QuantStudio™ DX will provide rapid, precise, and accurate results for a large number of molecular assays. Since the testing is performed in real-time, the Laboratory will be able to more realistically analyze possible outcomes and responses to potential healthcare crises.

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2. Espy, MJ. Real-time PCR in clinical microbiology: applications for routine laboratory testing. *Clinical microbiology reviews*. 2006; 19(3):165-256.
3. FDA-cleared RT-PCR assays and other molecular assays for influenza viruses [Internet]. Atlanta: Centers for Disease Control and Prevention. [Cited 2017 March 15]. Available from <https://www.cdc.gov/flu/pdf/professionals/diagnosis/table1-molecular-assays.pdf>.
4. QuantStudio™ Dx real-time PCR instrument. USA: Life Technologies; 2014.

**ISDH Laboratorian receives National Honor**

*Jessica Gentry with Dr. Ed Desmond  
(left)*

Jessica Gentry, the ISDH Laboratories' TB Supervisor, was the recipient of the Ed Desmond Laboratorian of the Year Award at this year's APHL/TB Controllers meeting. This award honors exemplary service, dedication, or leadership of a TB laboratory professional. The Desmond Award is a merit-based award, and Jessica was nominated for her contributions in data mining research, cost-effectiveness studies, refinement and enhancement of daily operation of the ISDH TB laboratory, and contributions to the National TB Laboratory community. This dedication was also recently acknowledged by the CDC, as Jessica was invited to provide bench-level expertise at the CDC's Diagnostic Mycobacteriology Course in 2017. The award was presented by Dr. Ed Desmond, Eric Hawkins (ISDH TB Controller), and Dr. Sara Blosser (ISDH Clinical Microbiology Director).



*From left to right: Dr. Ed Desmond, Jessica Gentry, Dr. Sara Blosser, Eric Hawkins*

**About The LAByrinth**

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