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Microfluidic Genotyping by Rapid Serial PCR and High-Speed Melting Analysis

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Guest:
Dr. Carl Wittwer is Professor of Pathology at the University of Utah Health Sciences Center.

Bob Barrett: This is a podcast from C*linical Chemistry* sponsored by the Department of Laboratory Medicine at Boston Children's Hospital. I am Bob Barrett.

Turnaround times are often long in PCR-based tests because multiple reactions are usually performed in parallel using programmable thermal cyclers. These methods typically use a single protocol, placing constraints on assay design.

A DNA sample may need to be processed multiple times to accommodate varying protocols for a panel of tests. As a result, use of consumables, turnaround times, and laboratory errors all increase, thus decreasing throughput.

A paper appearing in the October 2014 issue of *Clinical Chemistry* demonstrated a technique using High-Speed Melting Analysis, with Microfluidic Genotyping by Rapid Serial PCR that may change all that.

We are joined by one of the authors of that study, Dr. Carl Wittwer. He is Professor of Pathology at the University of Utah Health Sciences Center and also affiliated with ARUP Laboratories' BioFire Diagnostics, and is an Associate Editor of *Clinical Chemistry.*

Dr. Wittwer, what exactly is High-Speed Melting as it's applied to PCR?

Carl Wittwer: Well, in general, melting analysis is a way to analyze post-PCR products, just like a gel is a way to analyze what you have amplified or sequencing.

Now, what's better known is High Resolution Melting or HRM, which was initially developed around 2003, 2004, and has become known as an inexpensive, very rapid method for verifying PCR products.

Now, High-Speed Melting is something a bit different, because the melting rate, that is, if you imagine a double-

stranded DNA molecule; it's those two strands wound around each other in a helix, and the melting analysis is using heat until those two strands unwind or denature.

And with the use of fluorescent dyes that are fluorescent with double-stranded DNA but not single-stranded DNA, you can watch and quantify the melting process, both in terms of temperature, melting temperature or TM, and in terms of the shape of that melting curve.

And High Resolution Melting became popular because of the power of that very simple analysis that can be done on the same machine as real-time PCR machine.

So today most 80%, 90% of real-time PCR machines include what's called High Resolution Melting, albeit at different levels of resolution.

High-Speed Melting is a variant of High Resolution Melting that does the temperature ramp--the rate of heating--much faster than it's typically done.

So for instance, that might reduce the time required for melting analysis from an hour down to about a minute. The melting process itself is very amenable to being measured at high speed, and that wasn't entirely realized, and it's not present on most instrumentation, which would more typically take 15 minutes to an hour to do a melting analysis.

So it's just an issue of speed; how quickly can you analyze with melting analysis.

Bob Barrett: Do microfluidics lend itself to faster PCR cycling or so-called extreme PCR, and if so, why?

Carl Wittwer: Certainly, microfluidics, you have to consider macrofluidics, a volume of a milliliter. You can imagine that as a cubic centimeter in a test tube. And the aspect of microfluidics really is going down into micro or nanoliter or even picoliter volumes.

And one advantage of going down to the much smaller volumes, particularly in reactions that require changes of temperature, is that it's a lot easier to change the temperature of small volumes than large volumes.

So potentially at least you can change temperatures very rapidly with microfluidics. So the PCR process itself, you don't have to work as hard to get the sample to vary between 55, 75, and 95 degree centigrade, because you don't have to thermally move as much fluid.

Now, it's also true in terms of High-Speed Melting, it's a lot easier to work with smaller volumes. So microfluidics just allow you, again, to do both PCR cycling and High-Speed Melting faster.

Bob Barrett: Can High-Speed Melting immediately follow rapid PCR automatically without intervention?

Carl Wittwer: Yeah, typically these techniques, on some instruments they can be done right after each other. In some work that we have done, we have typically measured them separately. There are techniques for rapid PCR that don't allow melting analysis.

But in the article that we recently published in *Clinical Chemistry* one advantage of the system is it really is the first instrument that can both do PCR in the order of one to two minutes, and without any intervention or any other instrumentation, follow that immediately by High-Speed Melting.

So after the sample is applied onto the instrument, we are talking about three minutes or so to produce both the amplification of the product and its analysis by High-Speed Melting. So that I think is one aspect of this current publication that really hasn't been seen before.

Bob Barrett: How can High-Speed Melting analysis be automated to be used clinically?

Carl Wittwer: Well, that's a good question. Most instrumentation out there is not high-speed, and I even mentioned on High Resolution Melting for competitive marketing purposes all companies will say they have obtained that goal, but the quality of high resolution melting very much still depends on the instrumentation.

And particularly in the terms of speed, it's very typical for melting analysis to be performed on the order of an hour, and that's unnecessary and very, very slow.

So the High-Speed analysis of course is attempting to tell people that it's not the melting that's slowing your results down; it's your instrumentation.

So a typical instrumentation for both real-time PCR and melting is much slower than it needs to be. So the aspect of automating the High-Speed Melting is just being aware of this, either on the research setting or with eventually manufacturers, to realize you don't need to take a lot of time to get the High-Speed Melting data out.

The other component of course is to analyze the data with some sort of software, and that software is becoming more widely available, and any one who works with the high resolution melting needs something that will help them with the analysis. And getting the High-Speed Melting to be easily analyzed in software is something that is currently developing.

It's not typical to run melting experiments at the speeds of 0.1 to 1 degree C per second and the number of data points you would obtain off of that requires the instrument to rapidly collect the data, and the software really has to match that.

So the automation component is primarily one of integrated software that can make the scanning or genotyping calls of High-Speed Melting.

Bob Barrett: Well, finally doctor, talk about the difference between targeted and massively parallel analysis?

Carl Wittwer: Yes, a very interesting contrast. Today everyone has heard about the genome, the human genome, that we can sequence it for thousands of dollars and perhaps a thousand dollars in the future or even less, and all that has been made possible by techniques that I like to refer to as massively parallel analysis, also called next-generation sequencing.

But this massively parallel analysis allows you to do everything in terms of sequence analysis at once. So the highly parallel nature is what allows you to approach complete bacterial sequencing or even human genome sequencing, where you are talking about up to 3 billion base pairs.

And this is now feasible, not necessarily on a very rapid approach, because of the analysis at the end, but still we are talking days to get the whole genome out from one sample.

Now, that's very different from regular clinical diagnostics, where usually a clinician will think of a need for a particular lab test, a targeted lab test; if it's in genetics it would be a single base variant perhaps that's been associated with coagulopathies or a particular kind of cancer.

And you do a targeted analysis, most conveniently done by PCR and the primers of PCR to focus in on just one specific small area. And it's very different from the massively parallel techniques, which essentially sequence everything. And when you sequence everything, you do get a lot of results and a lot of base changes that you need to attempt

to interpret. So it makes the analysis component of sequencing to pull out relevant variants, which is actually very difficult.

That's a current challenge in massively parallel sequencing today; it's a totally different philosophy. The massively parallel approaches used to be considered and criticized as fishing expeditions. But now the pond is full of fish so there are so many things you can pull out quickly and easily that it's no longer really criticized. But it does present some difficulties in interpretation and analysis that's not present when you just focus in upfront on a very small target to answer a specific clinical question.

So that's changed the way we think about genomics, and it's changing the way that clinicians need to be aware of in terms of ordering patterns and interpretation. So it's a very dynamic process that we are going through

Bob Barrett: Dr. Carl Wittwer is Professor of Pathology at the University of Utah Health Sciences Center. He has been our guest in this podcast on High-Speed Melting Analysis and Rapid Serial PCR from C*linical Chemistry*.

I am Bob Barrett. Thanks for listening!