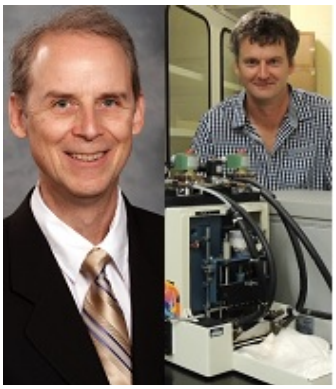
Clinical Chemistry

Extreme PCR: Efficient and Specific DNA Amplification in 15–60 Seconds



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Guests:
Dr. Carl Wittwer is Professor of Pathology at the University of Utah Health Sciences Center, and John Mackay is Technical Director of dnature diagnostics & research ("dna in a range of nature") in New Zealand.

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

Extreme sports, extreme eating, extreme weight loss, extreme makeovers, just when you think you've heard it all, how about Extreme PCR?

In the January 2015 issue of *Clinical Chemistry,* a Special Issue devoted to Molecular Diagnostics, researchers from the Department of Pathology at the University of Utah School of Medicine in Salt Lake City published just that.

They describe a technique for efficient and specific DNA amplification in 15 to 60 seconds. They found that by increasing primer and polymerase concentrations and decreasing cycle times to less than two seconds efficient high yield PCR from human DNA is possible in just 15 seconds, making it well suited to applications where immediate results may be critical.

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

Dr. Wittwer, first let's talk about PCR. What is PCR and Rapid-Cycle PCR and Extreme PCR? How are all these things different?

Dr. Carl Wittwer: PCR stands for the Polymerase Chain Reaction, a technique that was created, invented in around 1996. It is *in vitro* or test tube method for replicating a short segment of DNA in human or other organisms.

It's become one of the cornerstones for foundations of molecular biology because of its simplicity and elegance.

When it was initially developed back in the late 1980s, it was a slow process in that it would require typically maybe three to five hours to complete the reaction that's metered by temperature cycling.

The requirement of PCR was that you had to change the temperature of the reaction so that three different things would happen at three different temperatures.

At first you needed to denature, or separate, the two strands of DNA, if you imagine the double helix, you need to convert the double strands into separated two single strands. And that occurs at a high temperature typically somewhere around 90 to 95°C, so near boiling of water.

And the other two components of the test also occur optimally at different temperatures, so at an annealing temperature--an annealing temperature for primers that specifically focus and identify the bounds of a small segment of DNA--that typically occurs around 50 to 65°C.

And the final piece, that when put together creates the Polymerase Chain Reaction, is an intermediate temperature typically around 70 to 75° C where the polymerase, the actual enzyme, synthesize with more DNA.

So it's a three component process that has to be repeated typically around 30 times. Why it's so useful is that the amount of DNA replicated each cycle is approximately double. So after 10 cycles, 10 of these three-temperature cycles, you would end up with approximately a thousand copies. After 20 you'd end up with a million copies. And potentially after 30 cycles you would end up with a billion copies of your reaction components that were not completely consumed yet.

So, all of that being put together, the requirement of having three different temperatures, the solution for automation was to use heating blocks to do that, and it took time to change the temperature of a metal block and also to get this temperatures into the samples.

So what that meant is typically each cycle would take several minutes, not because it was required to take that long, but because of it took long to change the temperatures of the system, for what people were convenient with.

In around 1990, Rapid-Cycle PCR considered how much time was really necessary at each of the temperatures, each of the PCR steps. And what was found is that once you reach temperature, very little time it's actually required,

particularly at the top and bottom temperatures, at the denaturation, and the annealing temperatures.

In fact so little time is required that once you reach those top and bottom temperatures, you can immediately continue changing the temperature back to the next step, sort of a momentary denaturation or annealing temperature was really all that was necessary.

So, practically utilizing that, became known as Rapid-Cycle PCR. So that took the time down from hours, two to four hours down to 10 to 30 minutes, which is a significant advantage particularly if you have to wait for the reaction to complete to go on with your work. So the Rapid-Cycle PCR, specifically early 1990s increased the speed about an order of magnitude.

Now, Extreme PCR is a recent development that was first published text in the January issue of *Clinical Chemistry* in 2015. And it takes Rapid PCR and makes it even faster. So, if Regular-PCR took a couple of hours and Rapid-Cycle PCR to 10 to 30 minutes, you can get at least another order of magnitude and speed by going to Extreme PCR, which will amplify or complete 35 cycles and as little as a total of 15 seconds--typically 15 seconds to about a minute is the comfort zone.

So, we have taken a long process of hours, nearly gotten it down into seconds, by going through first Regular PCR and then that Rapid-Cycle PCR and then Extreme PCR. It's kind of interesting in the middle time period, in the early 2000s, there was something called Fast PCR, which was actually slower than Rapid-Cycle PCR, but usually refers to increasing the speed of amplifying very, very long products, whereas Rapid-Cycle and Extreme PCR typically focus on short products.

So it's all about timing and speed; that’s the progression between the original invention and making the reaction faster and faster. Of course PCR, being a cornerstone and foundation of molecularly diagnostic, means it’s used so often that having a means to do it faster can be very advantageous.

If you look at molecularly diagnostic applications in cancer, genetics, and infectious disease--for instance infectious disease—if you can get the answers out faster, means you don’t have to take as much time doing the test. So you start thinking about applications in bio-threats or infectious diseases where timely answers are really important; you start gravitating not from centralized laboratory testing that needs to factor typically overnight shipping into the turnaround time, but your analysis tends to get closer and

closer to the patient within the same hospital or even near to the patient, in which results could be returned very, very quickly.

Bob Barrett: So what happens to the sensitivity, specificity, and the yield as PCR becomes shorter and shorter?

Dr. Carl Wittwer: Very interesting question. Prior work had typically run into a blockade of decreasing quality as you increase the speed. So typically on most methods and instruments when you try to get down to faster and faster cycles, you start losing sensitivity, yield, potentially even specificity.

So that it limited people typically down to completing 30 cycles of PCR into the 5 to 10 minute range. And the previously fastest recorded PCR cycles that have been published have been in the 2-minute range, to actually detect a product.

But in almost all those cases, it's decreasing the quality of the analysis. So the interesting thing about Extreme PCR is that we were able to retain that specificity and yield and efficiency of amplification by a chemistry trick.

So it wasn't isolated instrumentation. We changed the components of the reaction; we changed specifically the concentration of primers and the concentration of polymerase, not by small amount, but by about an order of magnitude.

So by increasing primer concentrations and polymerase concentration about tenfold, ten to twenty-fold, we could decrease the time by that same amount, about an order of magnitude. And it makes sense if you think about it, primer annealing is, it's a second-order reaction of course, but it becomes a pseudo first-order reaction at high concentrations of primers.

So what that means is that if you double the concentration of primers, you would expect to have the required annealing time. So it's an inverse relationship between the concentration of primers and the required time for annealing. In a similar situation, although somewhat more complicated, it’s the same for the polymerase. If you supply more polymerase, particularly during the latter portions of PCR, you are able to complete the polymerization faster.

Bob Barrett: So doctor, what about the magnesium concentration, is that important for Extreme PCR?

Dr. Carl Wittwer: One other change that we also incorporated and found out during the process, what’s in magnesium is important higher concentrations of magnesium facilitate the very rapid

amplification of Extreme PPR, and that's predominately ,probably, due to increasing the annealing rates of primers.

So it's known in general that the rates of oligonucleotide annealing, of primer annealing, really depend on ionic strength, they are faster with higher ionic strength and particularly the divalent cations like magnesium that's already used in PCR, but seems to be critical for Extreme PCR to keep those annealing rates high enough to achieve the speeds of Extreme PCR.

Bob Barrett: Well finally doctor, why is Extreme PCR important?

Dr. Carl Wittwer: Well, doing things faster is important to some people and may not be important to others. For instance, one common comment when I embarked upon increasing the speed of PCR was, that gosh, do we really want the results faster? I'd like setting up my PCR reaction and going home for the night and relaxing until the next morning when the reaction was done.

So it certainly depends on the circumstance: when is it critical to receive a rapid result? Well, anything we ought to do sequentially in the laboratory, in research, if we need to do multiple sequential reactions, it's certainly advantageous not to take a couple of hours to do it each time.

If you can amplify those sequential reactions in less than a minute, you can get your entire scheme completed in well under an hour. In the clinical realm, of course, when is it necessary to provide rapid results? And that's where we tend to leave the central laboratory paradigm behind and go to rapid testing that becomes nearer and nearer to the Point-of-Care.

If you have to wait for the answers, it becomes very important to not have to wait very long. So imagine a situation where there is an outbreak of some infectious organism, we can use Ebola as an example.

And it becomes severe enough that not only do they monitor temperature and history to determine the likelihood of your having Ebola, but suppose that actual testing for the DNA of the organism became mandatory before crossing borders or before entering airlines. How long would you be willing to wait to get the test result back by standing in line?

And then the 5 minutes versus 30 minutes or a couple of hours makes a big difference and it even makes a difference between couple of minutes or a minute or 30 seconds.

So, that's the potential, all of this is done currently on experimental prototypes, but it does reveal that very rapid

PCR testing, even clinical testing, is completely feasible. So that's what it will be interesting to see whether we go this direction over time.

Bob Barrett: Thanks so much, Dr. Wittwer! Now, accompanying that paper on Rapid PCR technology, was an editorial whimsically titled "Taking it to the Extreme: PCR at Wittwerspeed." It was written by John Mackay, the Technical Director of dnature diagnostics & research in Gisborne, New Zealand, where his lab has developed PCR applications ranging from plant viruses and truffle species, to shellfish diseases and bee pathogens.

Mr. Mackay, you first started using PCR over 20 years ago. How has the speed of reactions changed over those past two decades?

John Mackay: Two decades makes it sound like a long time, but yeah, I think reactions have got a lot faster generally. So, many laboratories started out using reaction times and reaction tubes that meant reactions would take several hours and even elsewhere you go into some laboratories and you find there reactions are still running at similar times. They may have changed machines, but they have just stuck with the tried and true protocols.

Generally with faster ramping machines, faster enzymes, smaller reaction sizes, reactions have got faster. They are going from several hours down to typically 30 minutes to an hour.

Bob Barrett: In your editorial you mentioned that the speed of PCR now may affect your coffee break, but your paragliding? I think you have got a little explaining to do about that!

John Mackay: Yes, paragliding, well, that was back to days as a graduate student where I was fortunate to have an understanding boss after my graduate studies. So, it would mean after putting on a PCR run, complete with greasing the tubes and oil overlay as was common at that time, I'd also look out the window and if the wind was blowing the right direction, then I could walk home bring my paraglider in the car, 20 minute drive, and I would be soaring over beautiful Otago Beach in the South of New Zealand for couple of hours. Great way to unwind! I then land, back in car, back to the lab, and the PCR reaction would just be finishing, and so I could then perform the gel analysis. So, yeah with faster PCR those days have now gone.

Bob Barrett: As we say, "it's a nice gig if you can get it"?

John Mackay: Certainly it was in those days, certainly was in those days.

Bob Barrett: Increasing the concentration of the reagent sounds simple. Now this may be reminiscent of the original invention of PCR, but why do you think the work reported by Wittwer hasn't been done before?

John Mackay: That's a good question, and I think it comes down to a lot of challenging the dogma. I think there is a lot of dogma around PCR reactions and I think one thing that Professor Wittwer has done numerous times, has challenged the dogma.

And so one of these dogmas as I wrote in the editorial is that increasing, especially the primer and enzyme concentration, can lead to or will lead to non-specific reactions, that is, where you get undesired products or smears and the reaction when you analyze, it looks amiss.

But what Professor Wittwer and Dr. Farrar have done has been to do with two things in conjunction. That is, change the reaction chemistry and the speed simultaneously.

So I think that with PCR and molecular biologists, things have often been done linearly, which is, change one parameter at a time, and the dogma of the enzyme concentration and primer concentrations being within a certain range has been around for a long time.

And so I think it's just another instance of Professor Wittwer and his team, challenging the dogma around the PCR and molecular biology.

Bob Barrett: What do you think the broad implications of such rapid PCR reactions will be?

John Mackay: I think it's huge. I think will have a large impact on things like field biology, so there are number of field-based machines coming on to the market now. But these typically have lower throughput, and showing that such fast PCRs can be performed can potentially allow the Rapid Point-of-Care DNA diagnostics.

And perhaps not point-of-care but also field-based diagnostics, so things like Ebola virus of the 2014 outbreak, doctors, clinics, things like sexually transmitted infection, or STIs. Foot and Mouth disease is a particular concern here in New Zealand: we don't have it; we don't want it. So potentially you are not going to want samples to be transported around the country to a centralized laboratory for testing. If a very rapid and specific and sensitive DNA diagnostic could be performed in a matter of minutes, then that's going to have a huge impact on rapid diagnostics.

Bob Barrett: That was John Mackay, Technical Director of dnature diagnostics & research in New Zealand. Earlier in the podcast, of course, we heard from Dr. Carl Wittwer, who is Professor of Pathology at the University of Utah Health Sciences Center and is best known for work on Rapid PCR, LightCycler Technology and High Resolution Melting.

They have been our guests in the podcast from *Clinical Chemistry* on Extreme PCR. Their papers appeared in the January 2015 issue of *Clinical Chemistry,* a Special Issue devoted to Molecular Diagnostics.

I'm Bob Barrett, thanks for listening!