Clinical Chemistry

Influence of PCR Reagents on DNA Polymerase Extension Rates Measured on Real-Time PCR Instruments

Article:
J.L. Montgomery and C.T. Wittwer
Influence of PCR Reagents on DNA Polymerase Extension Rates Measured on Real-Time PCR Instruments.
Clin Chem 2014;60:334-340.
http://www.clinchem.org/content/60/2/334.abstract

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

Bob Barrett: This is the podcast from *Clinical Chemistry*. I'm Bob Barrett. Polymerase extension is an essential factor in the understanding of PCR and important in efforts to increase the speed of polymerase chain reactions.

In the February 2014 issue of *Clinical Chemistry*, the influence of PCR reagents on DNA polymerase extension rates were studied by examining nucleotide incorporation with DNA dyes. 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 is also affiliated with ARUP Laboratories, BioFire Diagnostics, and is an Associate Editor of *Clinical Chemistry*.

Dr. Wittwer, I'm sure most of our listeners know this, but what is PCR and what is the role of the DNA polymerase in PCR?

Dr. Wittwer: Excellent question. PCR has been around now since 1985. There's a Noble Prize associated with it. PCR itself stands for Polymerase Chain Reaction. It's an *in vitro* technique, that's a test tube technique that does what every cell in our body does, what it needs to, that is replicate, double the amount of DNA it has so it can split into two cells. And that's what PCR does in a test tube, and it does it actually faster than we can do it, than regular cells can do it.

Polymerase chain reactions can be completed very quickly so you can get over a million-fold amplification of a defined segment of DNA commonly done in 30 minutes or so. So, thus polymerase chain reactions become very useful over the past 30 years in molecular biology as one of the basic techniques to focus in on a segment of DNA and produce more products so it can be analyzed by different ways.

Now, the word polymerase suggests that a polymerase is very important in this technique called PCR. And it really is central, because the DNA polymerase is what makes more DNA. It's guided by nucleic acid primers that show the

polymerase where to start polymerizing -- that is making more DNA, and the progress is also controlled by temperature.

So to access DNA to replicate, DNA is usually first denatured at a high temperature, then the temperature is lowered down for primer annealing, and it's those primers that have annealed to the template that attract the polymerase that makes the actual DNA.

Bob Barrett: How is DNA extension measured, and why is it important to measure DNA extension rates?

Dr. Wittwer: Now, with such a central role in this reaction, measuring how much polymerase you have and how active it is should be a very important thing to know. And DNA extension rates and the activity of the enzyme of the polymerase usually are measured by assays that measure rates. And those in the past have been very complex measurements.

So, assays to measure how much of the polymerase is there usually required radioactivity and they end up telling you how much has been made from a complex template that actually isn't as simple as the PCR template. It's often done in conditions that don't resemble PCR.

So, if you really wanted an assessment of how much polymerase you had for PCR, you'd want an assay that was easy to use, didn't require radioactivity, and actually reflected, as close as possible, the conditions of the polymerase chain reaction itself, buffer conditions, temperatures, et cetera.

So, what we were able in this article to produce was a new assay that measures the DNA polymerase activity that has a lot of convenience to it that just uses dyes and a real-time PCR machine to measure rates or activity of the polymerase. That makes it much easier to study certain aspects or components of the PCR reaction and how they affect the actual polymerase activity.

Bob Barrett: Doctor, what effect do cations like potassium and magnesium have on polymerase extension?

Dr. Wittwer: What we found by using this assay, the polymerase assay, which can be done on common real-time PCR machines, 96, 384-well instruments, was that certain common things that are used in PCR actually hurt or decrease the polymerase activity. So, typically you would conclude that those components probably should not be in the PCR reaction for instance potassium, it's very common to include 50 mM potassium in PCR.

And what we found is that in terms of the extension rates of the polymerase, this actually decreases the rates by about 80 percent. So, you'd conclude that potassium probably doesn't help the reaction. It certainly doesn't help the polymerase.

Now, magnesium is a different story because it's a cofactor for the polymerase itself. So, whereas potassium hurts polymerase extension, magnesium often helps. And in terms of the polymerase extension rate, it actually increases until about 5 mM.

So, we're getting sort of a viewpoint of effects of different components on the PCR reactions and whether or not it makes sense to include those components. And something that people do everyday using common buffers often supplied by manufactures that don't always make a lot of sense in terms of the reaction itself or polymerase extension.

Bob Barrett: Doctor, what effect do DNA dyes, probes, and secondary structure have on polymerase extension?

Dr. Wittwer: Excellent question. For example, the DNA dyes and probes are often added to real-time PCR reactions so that the reactions can be observed in real-time by fluorescence. There is a price that is paid, however, in terms of polymerase extension when you add dyes or probes. For instance, adding a probe between the primers, it shouldn't be surprising that the polymerase would run into those probes and perhaps be slowed down. And, in fact, it is slowed down by probes between the primers.

Similarly, DNA dyes that are often used to visualize accumulating PCR product also slow polymerizations or the rate of polymerization. It differs according to different DNA dyes and their concentrations. Lower amounts of DNA dyes affect polymerase activity less. And as you would expect, secondary structure also inhibits the polymerization of DNA. Likely, a stronger secondary structures will inhibit PCR or, at least, slow it down more than templates without secondary structure.

So, those three findings are not too surprising except more people might not realize that adding things between the polymerase like probes or DNA dyes actually do slow down polymerase extension rates.

Bob Barrett: Well, finally Doctor, how do melting temperature depressors like glycerol and betaine and DMSO affect extension rates?

Dr. Wittwer: Also an interesting question because many commercial preparations for master mixes for PCR include enhancers,

things that for one reason or another may improve the yield or specificity of PCR.

Now, many of these actually just depress or lower the TM, the melting temperatures that typically have beneficial effects on the polymerase chain reaction. These include very commonly for instance DMSO, glycerol, betaine, less commonly formamide, all of which have an effect in general of lowering melting temperatures.

The answer on the effect of polymerase activity is somewhat interesting. Most of these PCR enhancers, the TM, melting temperature depressors slow down polymerase rates. With the exception of DMSO and, at least, on the templates that we have investigated, DMSO in low concentration of five percent to seven percent or so, increase the apparent rates of polymerization.

Now, if you think about why that might be, DMSO is often added to the polymerase chain reaction because of secondary structure. It tends to lower the affinity of the secondary structure binding, and to clear up those knots in front of the polymerase so that it's easier for the polymerase to actually extend through and complete the PCR extension.

In general, most of the PCR components, common PCR components that we think of tend to slow polymerization rates. Now, the exceptions are, of course, magnesium and in the case of DMSO, its effects on secondary structure as well. But many of these other components where they're headed in presumably to help PCR and polymerization do not appear to have much benefit. For instance, one other cation, ammonium sulfate, in our studies show a strong depression of the rates of polymerization.

So, you can't help but ask the question of why are many of these components added, they don't typically seem to help the reaction or at least help polymerization rates.

Now, it's true that there's much more to PCR than just the rate of polymerization, for instance, the fidelity of replication. And you can also ask the question of specificity and whether or not some of these added components are necessary for increased specificity.

They're all good questions and the focus of this manuscript, really, is to be able to study analytically the effect of these common additives and ingredients into PCR with the focus of making the polymerase chain reaction better and particularly faster. The speed has always been a strong interest of the authors of this manuscript. And we hope to push the time for PCR to much faster answers.

The rapid cycle PCR that we developed in the early '90s, we're revisiting that, which reduced the times down to about the 10 to 15-minute range. And what we're calling extreme PCR now looks at performing the same process in less than a minute. And it's interesting to many people that we can push the polymerization with the right ingredients and concentrations to produce high yield, highly efficient PCR reactions in less than a minute.

So, that's the general overview of the manuscript. And it has been my pleasure to talk with you.

Bob Barrett: Dr. Carl Wittwer is Professor of Pathology at the University of Utah Health Sciences Center. He's been our guest in this podcast on Polymerase Extension Rates from *Clinical Chemistry*. I'm Bob Barrett. Thanks for listening.