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Taking It to the Extreme: PCR at Wittwerspeed

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If the start times of PCR thermal cycling were plotted relative to frequency, we could expect to easily see sharp peaks of start times immediately before morning/afternoon coffee breaks, lunch times, and end of day, by operators safe in the knowledge that the amplification would take 1–2 hours to complete. Perhaps it is a little faster in many, but not most, diagnostic laboratories. Indeed, careful experimental planning when I was a student allowed leisure activities at opportune times "while the PCR was running." At the time, these PCRs were typically 50–100 μL in volume and used relatively thick 0.5-mL tubes.

In the late 1990s, the Guinness Book of World Records held an entry for "fastest DNA fingerprint" of approximately 11 min (essentially rapid real-time PCR detection). Further demonstrating that faster amplification concepts are not new, the term "rapid-cycle PCR" was coined in the early 1990s to define a thermal cycling program performed in 10–30 min *(1)*. The Guinness entry and rapid-cycle PCR have a common denominator: the world record was held by the original capillary-based LightCycler, and similar capillaries were used for the rapid-cycle PCR—both in the hands of Carl Wittwer from the University of Utah.

The work of Professor Wittwer and his team is no stranger to these pages, as they previously published real-time PCR applications on the LightCycler *(2)* as well as early papers on the now well-adopted genotyping and variant scanning methodology of high-resolution melting analysis (HRMA) developed in his laboratory *(3, 4)*—work that more than justified Wittwer as the subject of an "Inquiring Minds" article, also in *Clinical Chemistry (5)*. Since these developments, he has returned to his first molecular interest: increasing the speed of PCR.

Total PCR analysis time has decreased largely because of the advent of real-time PCR and now digital droplet PCR, in which gel electrophoresis bands have been replaced with amplification curves and scatter plots. Yet the major time component in the PCR itself is the thermal cycling time. Recent efforts to decrease these times have used chip-based reactions using microvolume reactions in <6 min *(6)* or a rapid-cycling instrument with high convective heat transfer in <3 min *(7)*. New companies are heating the reactions on particles (rather than heating reaction volumes) as a means to increasing ramp rates (the speed at which reactions heat and cool among the various temperature cycles) *(8)*. Despite these newer methods, most have yet to reach the commercial market, and none have come close to penetrating the dominance of the slower Peltier-based technology.

Recent work in Wittwer's laboratory has characterized the effect of PCR buffer and additive components on DNA polymerase activity using a novel nonradioactive assay *(9)*, thus paving the way for further improvements in PCR. In this issue of *Clinical Chemistry*, Farrar and Wittwer return to these original reaction capillaries (thinner than the current LightCycler capillaries and thus allowing faster heat transfer to the reaction volumes) and describe their concept of Extreme PCR, pushing down the time of amplification to <15 s—as little as 14.7 s for 35 cycles, in fact *(10)*. Such short times demand better accuracy.

Having frequently challenged the dogmas that existed in PCR and DNA melting kinetics, Wittwer's laboratory has overturned the belief that higher primer and enzyme concentrations always lead to nonspecific amplifications. That belief is true if typical thermal cycling rates are used; but under extreme PCR conditions, the amplifications result in sharp specific bands (and melting peaks), presumably because of a lack of time for off-target products to be generated. This increased specificity was also observed for certain targets in the original rapid-cycle PCR of 10–30 min compared with thermal cycling of 2–3 h *(1)*. Extreme PCR has also used existing enzymes successfully, as well as newer-generation highly processive systems. These current enzyme systems have been successfully used previously in reactions of <3 min *(7)*.

Thus the extreme PCR technique requires 3 main parameters to ensure the robust, efficient, and sensitive amplifications we typically enjoy: rapid temperature cycling (very rapid!), higher primer concentrations (typically 20-fold higher than what would be considered usual), and higher enzyme concentrations (approximately 15-fold higher). Standard enzymes are less expensive now due to expiration of the *Taq* patents, and thus the chemistry modifications of extreme PCR look to have little

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effect on reagent costs. Interestingly, the reactions do not use any hot-start chemistry, noting the limitations of antibody amounts required and chemical modification activation times. Reaction setup on ice would be of limited practical use in the field, and instantaneous technologies such as aptamers would appear to be an attractive means of obtaining hot-start activation *(11)*.

Amplification (and concurrent real-time fluorescent detection) in less than a minute highlights the considerable future potential of PCR. Although it is a mature technology, the wider prospect of field-based PCR is only starting to emerge in small field-based instruments (some field instruments having been made available previously to military or specialized agencies). However, the throughput vs time-to-result considerations of current instruments may limit their utility with relatively low sample size and run times of 30–60 min. The ability to perform extreme PCR on a field-based instrument will obviate the need for high-sample-number capacity. Such an instrument could be rapidly deployed in biosecurity cases for dangerous diseases or organisms where suspect samples may preferably be quarantined and prevented from travel to a testing laboratory. Extreme PCR run times further demonstrate the potential for point-of-care diagnostics in areas ranging from rapid viral diagnostics (such as in the 2014 Ebola outbreak) to testing and immediate treatment for sexual health patients with *C. trachomatis* infections—patients who often miss treatment due to time delays between samples taken and test results being made available. The rapid implementation of HRMA for genotyping could allow a patient to be tested for a susceptibility variant immediately before receiving a medication, to ensure appropriate efficacy.

The demonstration in this issue's report shows the speed at which amplification can occur, traditional speeds once again being limited by instrumentation. It uses rapid switching between 2 water baths (once more, harking back to the early days of PCR). It will be of great interest to observe the instrumentation developed that exploits this new technique: whether samples will indeed switch between steady temperatures or whether the sample can remain in a static position, as with most current instrumentation, and yet be rapidly heated and cooled in a reliable fashion using, for example, lasers *(8)*, infrared light *(12)*, or induction.

Although the time for testing of nucleic acid samples has decreased because of real-time PCR, and now vastly shortened cycling conditions have been shown with extreme PCR, the effort and time in nucleic acid extraction becomes further highlighted. A number of rapid extraction methods exist, using novel enzymes now commercially available *(13)* or high-efficacy 1-tube extraction buffers *(14)*. However, many of these systems have not been characterized for applications such the high-sensitivity requirements of pathogen detection. Other options—again, for applications in which targets are in abundance—may be the "direct PCR" enzyme and buffer systems that work directly with the sample material, removing the need for nucleic acid extraction altogether. However, whether these system enzymes and buffers have the processivity for extreme PCR remains to be seen.

So we now look forward to the meeting of extreme PCR with superlative sample extraction. The days of the coffee break (or paragliding stint) while the PCR thermal cycling runs may soon be over. But in the time it took to read these words, your nucleic acids results are in and a patient is already being treated.

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