Clinical Chemistry 55:4
833–834 (2009)

Citation Classic

The Beginnings of Real-Time PCR

P. Mickey Williams[1*]

Featured Article: Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. Genome Res 1996;6:986–94.[2]

During the mid-1990s the PCR was becoming a mature technology, impacting such areas such as cloning and automated sequencing. Many scientists were making efforts to tame the quantitative power of PCR. The power of exponential amplification was tremendous, but it had proven hard to control in quantitative applications. Many attempts were made to use so-called end-point quantification. This approach attempted to determine a starting target concentration based on the quantification of the final amplified product. Several aspects of PCR complicated the success of these efforts. A major source of difficulty was that PCR often reaches a plateau stage at which exponential amplification ceases and there is very little to no further product accumulation. Therefore, the product concentration at plateau is similar for all starting target amounts. In essence, it is impossible to accurately quantify starting target concentrations if PCR reaches plateau. Another complication is that PCR, like any enzyme-driven reaction, is sensitive to reaction inhibition. Many of the most interesting biological samples and methods for nucleic acid purification possessed known inhibitors of PCR (e.g., heme from blood). Southern and Northern blots with radioactive probes were the method of choice for many aspiring scientists wishing to have semiquantitative nucleic acid data, although these methods came with their own inherent pitfalls.

It was during this time period that new thoughts began to surface. David Gelfand and Pam Holland and colleagues at Cetus had described the 5′-to-3′ nuclease activity of DNA polymerase and the method they used to harness this activity to degrade a hybridization probe placed in a PCR *(1)*. Their discovery was eventually and cleverly named "TaqMan," honoring one of the first electronic video games, Pacman. Additionally, Russ Higuchi and colleagues, formerly from Cetus but now at Roche, described "simultaneous amplification and detection" of PCR products achieved by use of the accumulation of ethidium bromide fluorescence generated during a PCR *(2, 3)*. Ken Livak and colleagues at ABI described the use of fluorescent energy transfer for generating a signal system for probe degradation in the 5′-to-3′ nucleolytic PCR assay *(4)*. Major advances in PCR equipment were also taking place. A group at Roche led by Bob Watson developed a kinetic PCR thermal cycler that captured real-time EtBr fluorescence. Another group, led by Carl Witwer and Kirk Ririe, developed a capillary-based rapid thermal cycler that dramatically decreased times required for PCR reactions.

It was during this period that Roche's efforts to bring PCR technology into the diagnostics arena began to bear fruit. A recently described disease, AIDS, was the major focus of the efforts of many researchers and pharmaceutical companies. A clear and powerful application of PCR was in the detection and quantification of HIV, the causative agent of AIDS. PCR afforded a sensitive detection method. Shirley Kwok, John Sninsky, and colleagues at Roche developed a quantitative reverse-transcription PCR test for measuring HIV viral RNA copy numbers. The use of a coamplified control sequence of known copy number and detection before plateau phase helped overcome some of the hurdles to successful PCR quantification. This test eventually became the Cobas Amplicor HIV-1 Monitor test. The development of this test was a pivotal moment in bringing the power of PCR technology to diagnostic applications. The test has been used as part of many clinical studies and has clearly demonstrated that viral burden is an important end-point of therapy response.

During this period many additional quantitative PCR advances were made; an example is the work of Mike Piatak and his colleague Jeffrey Lifson. They too were focused on HIV viral burden and they described another means of employing PCR for the quantitative detection of HIV. They published a report of the development of a quantitative competitive PCR as a tool for monitoring HIV viral burden *(5)*.

My laboratory at Genentech was fortunate enough to have connections with both Applied BioSystems and Roche. We were selected as the β test site for a prototype real-time quantitative PCR instrument being developed by Applied BioSystems. Both Chris Heid and I realized the potential of this application if it truly provided sensitive, reliable, and accurate quantification of

[1] Roche Molecular Systems, Pleasanton, CA.
* Address correspondence to the author at: Roche Molecular Systems, 4300 Hacienda Drive, Pleasanton, CA 94588. E-mail mickey.williams@roche.com.
[2] This paper has been cited 2895 times since its publication.
Received January 29, 2009; accepted February 4, 2009.
Previously published online at DOI: 10.1373/clinchem.2008.122226

target nucleic acids. With the successful development of this instrument, researchers would have a powerful tool to address any nucleic acid sequence or sample type of their interest. We began the initial experiments, during which we were learning how to design primers and probes for best results on this prototype instrument. After several months of close interactions with both Applied BioSystems scientists and engineers and our colleagues at Roche, we knew this technology was real and ready for use. We designed a series of experiments to demonstrate to ourselves and others the potential of this tool. When we completed of these initial experiments we knew it was important to share the results with others in such a way that the technology would be used. This effort resulted in the publication of 2 articles in *Genome Research (6, 7)*, 1 of which is the article featured here. Never in our dreams did we realize the ultimate impact this methodology would have on the quantitative study of nucleic acids in both research and diagnostic applications. To date thousands of reports have been published describing research that relied on the sensitivity, dynamic range, and precise quantification of real-time PCR and reverse-transcription PCR. Many have considered this technique the gold standard of sequence-specific nucleic acid quantification. Since our early work, many have improved upon the early efforts, and today a researcher has many instruments from which to choose. Looking back, it was a great pleasure to have been a part of this technology in its infancy and to watch it grow into the powerful tool it has become.

Finally, to highlight the link between our 1996 article and the journal *Clinical Chemistry*, it should be noted that our work was presented at the AACC Oak Ridge Conference a year earlier in 1995, and the abstracts published in *Clinical Chemistry (8, 9)*.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures of Potential Conflicts of Interest: *No authors declared any potential conflicts of interest.*

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

References

1. Holland PM, Abramson RD, Watson R, Gelfand DH. Detection of specific polymerase chain reaction product by utilizing the 5′-3′ exonuclease activity of Thermus aquaticus DNA polymerase. Proc Natl Acad Sci U S A 1991;88:7276–80.
2. Higuchi R, Dollinger G, Walsh PS, Griffith R. Simultaneous amplification and detection of specific DNA sequences. Nat Biotechnol (Lond) 1992;10:413–7.
3. Higuchi R, Fockler C, Dollinger G, Watson R. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Nat Biotechnol (Lond) 1993;11:1026–30.
4. Livak KJ, Flood SJ, Marmaro J, Giusti W, Deetz K. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Appl 1995;4:357–62.
5. Piatak M, Jr., Luk KC, Williams B, Lifson JD. Quantitative competitive polymerase chain reaction for accurate quantitation of HIV DNA and RNA species. Biotechniques 1993;14:70–81.
6. Gibson UE, Heid CA, Williams PM. A novel method for real time quantitative RT-PCR. Genome Res 1996;6:995–1001.
7. Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. Genome Res 1996;6:986–94.
8. Heid C, Stevens J, Williams PM. Quantitative PCR using closed tube multicolor fluorescence. Clin Chem 1995;41:1686.
9. Gibson U, Heid C, Ling A, Stevens J, Williams PM. Quantitative RT-PCR using closed tube multicolor fluorescence. Clin Chem 1995;41:1686.