I Basic technologies

1 Real-time polymerase chain reaction

Mickey Williams

The invention and successful practice of the polymerase chain reaction (PCR) by Kary Mullis and colleagues in 1983 set the stage for a scientific revolution. PCR established a base technology from which many specific and diverse applications have grown. PCR has played a crucial underlying technological role in many aspects of the genomic age that we experience today. The power to assess complete genomic sequences starting with minuscule amounts of target molecules entrenched PCR as the backbone of many subsequent analytical techniques. The sequencing of the genomes of many diverse species and the ability to discriminate individuals within a species have relied on PCR as an instrumental component.

The knowledge of genomes has led to the ability to identify sequences representing the coding genes that carry the blueprints for the construction of proteins. It is of great scientific interest to study the regulation of these gene-encoding messenger ribonucleic (mRNA) molecules. The study of gene expression has led to a better understanding of different biological states that exist within different tissue types, reflecting their different functions. Gene expression changes provide insight into underlying molecular and functional differences that exist between diseased and normal tissues. PCR has had a profound impact on gene expression studies as well. In 1991, while I was a junior scientist at Genentech, my scientific life was intensely affected by PCR. I was part of a team charged with developing assays to assess clinical outcomes of a vaccine treatment for human immunodeficiency virus (HIV) infections. I became aware of PCR and reverse transcriptase (RT)–PCR as means of quantifying specific sequences found in biological samples and was fortunate to meet some of the best and brightest PCR gurus at Roche Molecular Systems, born from Cetus Corporation, where Kary Mullis had worked. I also was introduced to another equally brilliant group of scientists from Applied BioSystems Inc. (ABI), which had gained research rights to the PCR patents. ABI

had some exciting research instruments under development. My introduction to these companies and scientists occurred during the period when PCR became a powerful tool for the quantitative assessment of gene expression. Following are some of my recollections of the time preceding and leading to the introduction of real-time quantitative PCR (qPCR).

EARLY DAYS

During the mid-1980s, I was a part of the growing number of postdoctoral trainees and scientists exploring gene expression as a means to gain insight into differentiation of tissues and the functional workings of cells. Specifically, I was studying the mechanisms by which embryonic fibroblasts could differentiate into fat (adipose) cells. The art of gene expression analysis was laborious, relying on such techniques as construction and screening of complementary deoxyribonucleic acid (cDNA) libraries, subtractive library screening, northern blots, and of course lots of cell culture. Considerable effort was needed to establish the infrabreak;structure for gene expression experiments. My end goal was to correlate changes in gene expression with biological changes within the cell during the differentiation into adipocytes. Many scientists were using similar approaches for their biological quests. Experiments were laborious and time consuming, often taking weeks to obtain results – provided that all of the technical aspects worked well. Oftentimes a flawed reagent or careless mistake meant weeks lost. An integral part of this effort was the use of copious amounts of radioactive labeling compounds needed to detect sequences of interest. I remember that one of the key elements of our experimental planning was the shipment schedule of the radiolabeled nucleotides. We all wanted to have the freshest batch of ^{32}P-deoxyribonucleotide triphosphate (NTP) for our "important" experiments. These were indeed fun times!

Molecular biologists, using the tools of nucleic acid hybridization, cloning, and sequencing, were busy discovering new transcripts and gene sequences. This was the time when discovery of a new gene or its transcript was often the serendipitous result of an unexpected band on a northern blot or a colony detected in a screening experiment. The use of nonstringent hybridization and washing conditions would permit related but nonidentical sequences to "light up" with radioactively labeled hybridization probes. Researchers who followed up and identified these new transcripts (or genomic sequences) often immediately wanted to learn the tissues of action for these uncharacterized genes. Beyond discovering the cells and tissues of expression of these newly discovered genes, efforts were launched to understand the regulation of this gene's expression as it correlated with such biological state changes as differentiation, cell stimulation with growth factors, and disease. Another active endeavor was the understanding of gene transcriptional regulation. These were the days of promoter bashing (deleting various DNA segments upstream of a gene to determine the impact on transcriptional regulation). These were also

the times during which the gel-shift assay was used for identifying the transcriptional regulatory proteins.

Interestingly, these were also the times when nothing more than the detailed description of unexpected bands on a gel led to assured acceptance of a manuscript. Northern blots were the workhorse technology for these activities. Originally most northern blots used agarose gels that included formaldehyde and lots of meticulously and painstakingly prepared RNA (10 to 20 μg), ethidium bromide, nitrocellulose filters, copious amounts of precisely cut paper towels, seal-a-meal bags with a radioactive seal-a-meal instrument (left behind Plexiglas shields), radioactive probes, x-ray film, boxes of latex gloves, and weeks of time. It was not uncommon to develop a film after weeks of effort to realize that one of many possible glitches had impaired the results. I vividly remember one experience from which I learned that plastic wrap sloppily left sticking out of an x-ray film cassette permits light to leak onto the film. This event ruined weeks of waiting for the perfect film image. So it was back to the beginning of the experiment oftentimes after learning such lessons.

THE STORY UNFOLDS

To obtain quality data it was *very important* to begin with the best quality RNA. Many of us remember the first time we were trained in the art of RNA preparation. Many laboratory rules were devised and often posted to prevent degradation of the much-sought-after prize of high-quality full-length RNA. We were taught to use only oven-baked glassware and diethyl pyrocarbonate (DEPC)-treated water, always wear sterile gloves, use only pipettes dedicated to RNA, never open a tube of the dreaded RNase enzyme on a bench where RNA would be purified, never use a pipetteman used for dispersing RNase for purification of RNA, and so on. Although we still follow strict protocols, the introduction of many commercial kits for purification of total RNA has made this a more reliable and less stressful aspect of routine laboratory practice. I do not think many scientists today experience the anxiety that many of us "senior" scientists felt prior to the isolation of RNA from a large and important experiment.

As the study of gene expression continued to be a focus for many experiments, many technical improvements came into the picture. Soon we replaced the messy seal-a-meal bags with glass tubes and hybridization ovens. Brittle and flaky nitrocellulose found competition from more flexible nylon membranes; radioactive labeling techniques were challenged by nonradioactive chemiluminescent approaches. Many new enzymes and tools were harnessed for amplification and labeling of probes. The introduction of riboprobes added a means of producing high-specific-activity RNA probes, which permitted sensitive detection of low-level transcripts. The forerunner of microarrays, the dot blot, was introduced. Dot blots did not permit the visualization of transcript size as did northern blots, but dot blots afforded easy multiwell experiments in which many samples or probes could be analyzed simultaneously. Ninety-six-well dot blots did not

quite parallel the massive standards of today's microarrays, but they were a long step beyond 12-well northern blots.

One of the most prevalent dilemmas of the era of northern and dot blots involved the aspect of quantitative assessment of transcript expression levels. It was of great interest to document the expression changes in mRNA levels resulting from biological state changes. Many issues needed to be overcome to permit meaningful quantitative assessment: (1) the painstaking task of preparing good-quality RNA, (2) how to detect and quantify the amount of a given transcript, and (3) how to normalize the load from well to well. This was an era of scientific art during which many creative attempts were made to address these topics. For detection and quantitation of mRNA transcripts, many researchers relied on densitometer analysis of x-ray film images as a means of adding quantitative values to the intensity of the northern blot bands or dot blots. This technique required efforts to ensure that all measured quantities were within the dynamic range of accurate measurement. A saturated image would obviously lead to lack of quantitative results. I believe that some did a much better job than others at attempting to understand and apply these techniques in the best manner. For sample-to-sample comparisons, utilization of additional transcripts termed "housekeeping genes" permitted sample-to-sample normalization for the amount of total RNA loaded. These housekeeping genes represented a class of genes needed for essential metabolic functions in all cell types. It was believed that expression of this class of genes would remain constant as their functions were essential for basic functions of all cells. During this period, many data were published that relied on such housekeeping genes as β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for sample normalization. Often the bands on a northern blot were so bulbous that one could be assured that the image was saturated and accurate measurement was not feasible. Such saturated bands are clearly not proof of equal sample loading. Nevertheless, this was the state of affairs during these days. The art of quantification produced many valuable insights that kept the science moving forward. It was in this backdrop that PCR entered our lives.

UNDERSTANDING THE PCR REACTION

Shortly after the introduction of PCR for the amplification of DNA, the addition of reverse transcription added the ability to amplify RNA via a cDNA intermediate. RT–PCR was born. The original methods used two independent steps, whereas reverse transcription was done prior to PCR amplification. Now we have been provided with blended enzyme mixtures or even single, dual-activity enzymes that permit the process of RT–PCR to proceed in a single unopened tube. It was not long after scientists began applying PCR and RT–PCR when the logical extension was made to use this technology for the quantitative assessment of the starting template. Many researchers made early attempts at quantifying the starting target by running PCR products onto a gel and using techniques to measure the amount of product generated. The notion that more initial target would generate more product (as evidenced by a darker stained band on a gel) was true, to

an extent. However, it was soon made clear by the PCR gurus that PCR assays eventually cease to produce exponential gains with each successive cycle. The understanding of the PCR plateau put an end to the simple gel-based methods of qPCR. Simply put, the initial excesses of enzymes and primers that exist at the beginning cycles of PCR soon become limiting as excess amounts of product are generated. At this point there is more PCR product than reagents available in the tube for the next round of amplification. When this occurs in later PCR cycles, the exponential increase in product per cycle is lost. Eventually, as the PCR continues, a cycle is reached at which little or no product is generated. It is during this initial plateau phase and final plateau that quantitative measurements are confounded. One fix to this phenomenon was short lived: One used a serial dilution of input target and measured the correlation with dilution of target and the target accumulation. One could use linear analysis to choose a range of input targets that resulted in linear output of product and estimate the input target quantity across samples. A similar approach used stopping the PCRs every couple of cycles throughout the assay. A small sample would be removed for gel analysis, and, again, early preplateau products could be compared for a semiquantitative assessment of input target quantity. These approaches required normalization, and housekeeping gene analysis was used for each sample. The excessive sample manipulation required with these early approaches was conducive to the dreaded PCR contamination, which could be nearly impossible to stop once started. Because such excessive amounts of product are generated during the exponential amplification, it was easy to contaminate clean reagents and also samples that should not contain the target. Tracking the source of PCR product contamination is often difficult and usually results in destroying all reagents and sometimes even changing labs. These early attempts at qPCR or semiquantitative PCR clearly resulted in cumbersome experiments that were almost not worth the effort. Northern blots were still frequently used as the method of choice.

If it were not for the exquisite sensitivity and rapid commercialization of PCR, this could have been the end of the story for qPCR applications. Researchers in the area of HIV and other infectious disease specialties realized the power of the sensitivity and impact that PCR would have on their fields of study. It was in this arena of infectious disease quantitation (especially HIV) that the next major improvements were made to bring qPCR and RT–PCR into everyday practice.

QUANTIFICATION PROSPECTS

My postdoctoral studies ended, and I accepted a job offer at Genentech, where my first project was to help develop a quantitative assay for measurement of HIV infections. Genentech had launched a two-pronged clinical effort to study the effects of HIV vaccines in preventative and therapeutic applications. The field of HIV research was entrenched in the use of CD4 cell counts as a surrogate end point of disease status, but interest was growing in the use of quantitative viral load as another possible surrogate. It was clear to many researchers that the

application of RT–PCR brought exquisite sensitivity to detect and quantitatively measure viral load. I was fortunate that our project leader, Jack Nunberg, had a connection to Roche Molecular Systems as he had come to Genentech from Cetus. It was a new beginning for me as I was introduced to many of the PCR gurus who had developed and commercialized this technology. It was my good fortune to have had the opportunity to work and learn from such people as David Gelfand, Russ Higuchi, Shirley Kwok, John Sninsky, and Bob Watson (to name a few). As it turns out, these scientists had already initiated a development of a U.S. Food and Drug Administration (FDA)-approved test for quantitative RT–PCR analysis of HIV viral burden. Their approach used a technology termed Amplicor™. This assay proved to be a workhorse in support of a multitude of clinical trials searching for therapeutically efficacious treatment of HIV, and has proven to provide a sensitive and accurate means to detect HIV in blood. Although these early trials did not yield a successful treatment, we had witnessed the birth of a powerful new assay tool, quantitative RT–PCR. We all were encouraged by the improvements in this technology and were motivated to help establish these techniques in routine laboratory research as well as in clinical research.

At the same time we were working with the Amplicor methodology, others (i.e., Jeffrey Lifson and Michael Piatak) described a gel-based approach.[1] It was called, among many names, "quantitative competitive" RT–PCR. The central component of this approach was the design and use of a competitor molecule that was spiked into the sample at known serial dilutions. The critical aspect of the competitor design was that it included the sequence for the same primers used for the target molecule of interest. The resultant competitor product amplicon needed to be a different length (or internal sequence) such that it could be differentiated from product in gel electrophoresis. The most critical aspect of this competitor was the demonstration that the PCR efficiency (i.e., how much product is generated with each successive cycle) was identical for both the target sequence of interest and the competitor. Demonstration of equivalent PCR efficiency required some assay development during which a series of mixed concentrations of competitor and target were tested to demonstrate expected ratios. The advantage of this approach when compared to all previous methods was the ability to run reactions to any end cycle and still obtain quantitative results. Even if a reaction was run into the plateau stage of PCR, the ratio of products from the target of interest to the competitor product was maintained from the starting sample throughout the entire assay into plateau. The assays were simple to run and interpret. A serial dilution of known amounts of competitor was put into replicates of the sample of interest or vice versa where the competitor concentration was held constant and the sample diluted. The PCR assay was performed, and results could be read from a gel. Line equations for target and competitor were made, and the quantity of the target was determined by comparison with the known amounts of competitor. After the technique was published, this approach was used by many researchers beyond the infectious disease arena. This approach was quickly adopted by scientists studying cell-based gene expression.

The application of PCR technology during this time was growing and impinged on science in multiple arenas. As a result of a strong PCR patent portfolio

protecting the rights of the inventors, many PCR work-around techniques began to blossom. Techniques such as strand-displacement amplification, self-sustained sequence replication, and so on began to flourish in the literature and at conferences. Many of these alternative methods had clear potential, but only a few of these competing technologies are still used to any extent for routine research applications.

REAL-TIME PCR

Several hallmark studies began to lay the groundwork for the soon-to-be-described real-time PCR. It was during this time in 1991 when David Gelfand and his colleagues described TaqMan™ methodology.[2] In that article, the use of a radiolabeled hybridization probe designed to hybridize to a sequence within the amplicon was introduced. During the reaction, the DNA polymerase would displace the radiolabeled probe and nucleolytic activity in the DNA polymerase would cleave the probe. In the conclusions the authors stated that the amount of probe cleavage correlates with the amount of product accumulation and hence correlates with the starting target amount. Another major event occurred in 1993 when Russ Higuchi, working with Bob Watson, demonstrated the quantitative accumulation of PCR product with a simple cycle-by-cycle ultraviolet (UV) box visualization of reactions containing ethidium bromide.[3] As more product was generated, the tubes accumulated more ethidium-derived fluorescence. A remarkable photograph of tubes on a UV box clearly demonstrated the concept. The final piece of the puzzle came from efforts by Ken Livak and colleagues at Applied Biosystems. They were making hybridization probes that contained two fluorescent dyes. One dye was a reporter dye, which was quenched in the intact oligonucleotide by a second dye (quencher dye) that by fluorescence resonance energy transfer (FRET) accepted the energy from the reporter, preventing reporter emission of light. Upon polymerase cleavage of the oligonucleotide probe, the quencher was no longer spatially in close proximity to the reporter and the reporter fluorescent light was now detectable.[4] It was the collaborative efforts of these scientists that led to the birth of real-time qPCR. I was fortunate to be collaborating with both groups during this period and was permitted to be the beta-test site for the first ABI real-time PCR instrument. This was a very exciting time. I think we all realized how important this technology would become. We had lots of fun during this period. Virtually every experiment gave us insight into the technique. A tremendous additional advantage of this technique was the closed-tube format. When a reaction was prepared and the tube was sealed, there was not a need to open the tube after the reaction. This technique reduced the potential for product contamination that was prevalent with competitive PCR gel formats. Our first assays were painstakingly developed as we often used a dilution series of probe and primer concentrations to optimize the reaction and obtain the most robust results. I remember the very first real-time experiment I ever ran; about halfway through the run, the power was interrupted and the instrument crashed. The experiment ended without results. After that

we soon invested in an uninterruptible power supply (UPS) unit. We also were lucky to have an engineer from ABI, Bob Grossman, personally on call for unexplained phenomena. Eventually, with the efforts of many, we began mastering the technology. These initial efforts resulted in two publications by Chris Heid et al.[5] and Ursula Gibson et al.,[6] which contained the first descriptions of real-time PCR and RT–PCR, respectively.

After the commercial launch of the ABI Model 7700 we began to add more instruments to our research group. This platform soon became a workhorse for many research projects. I was given an opportunity to speak at many conferences during this time period and was truly inspired by the potential power that this new tool would contribute to scientific research. I was in awe of the quantitative dynamic range, which was close to 7 logs of input target. The precision of well-designed assays was astonishing. Additionally, because this was based on PCR, the sensitivity was excellent. Soon there was an explosion of the use of this technology, with many others making significant contributions to its use and expansion. As with any new technology there was a learning curve, but soon a community of experts began to grow. Many other companies came into the arena as suppliers of real-time instruments, reagents, and kits. One of the more important applications came in clinical studies. Many researchers used the enormous sensitivity and quantitative data to study a variety of medically related topics. Quantitative pathogen detection and monitoring comprised many of the early clinical uses. One application that made a lasting impression on me was the first description of real-time PCR monitoring of minimal residual disease. The sensitivity of this technique clearly added to the ability to detect cancer-related chromosome translocations in the blood or bone marrow of leukemia patients.

It had always been a goal for the developers of this technology to use multiplex capabilities of instruments and dyes to add more genes to the analysis in the same tube. Although multiplexing is still not routine, many researchers have taken advantage of this aspect to include normalization genes in the same tube as the gene of interest. Another important advance in real-time instruments came as high-density thermal blocks were introduced. Today it is common to find 384-well blocks available. Some companies have moved to microfluidic devices and have increased the number of individual assay chambers to 1,536.

Today, real-time PCR is in routine use for research and clinical applications. Recently a breast cancer recurrence prediction assay was introduced that uses real-time RT–PCR (OncoType Dx; Genomic Health). This test is recognized by many oncologists as a valid tool to assist in patient management. Research efforts in oncology often rely on formalin-fixed paraffin-embedded (FFPE) archival samples. The processes of fixing and archiving contribute to degradation of RNA quality, often resulting in fragmented RNA of an average size of 150 to 200 bases. A strength of RT–PCR is that small amplicons can be designed such that even poor-quality fragmented FFPET samples are amendable to quantitative assessment. Real-time PCR is an accepted standard for many projects and has been approved for use in in vitro diagnostic assays by the FDA. In the early days of gene expression analysis with microarrays, it was common to verify microarray results with follow-up real-time PCR assays. It is well known that the dynamic

range of real-time PCR is much greater than that of microarrays. Real-time PCR has become the gold standard of quantitative nucleic acid analysis.

SOME FINAL THOUGHTS

As I reflect on the power of real-time PCR, I remind myself that good assay development is critical to success. It is important that the normalization genes are carefully chosen and validated. As we have learned, there is probably no one gene that is invariant in all biological situations. Therefore, selection of the best gene or genes is critical for data interpretation. Assays should be assessed for precision of technical replicates. If one would like to demonstrate that a twofold difference in gene expression is meaningful, the assay should have sufficient precision to statistically discriminate this difference. The linear dynamic range of quantitation should be explored. A sample is best analyzed for quantitation if it falls within this range. The impact of biological matrices is of great importance. It is known that such things as heme found in blood can inhibit PCR polymerases. Hence, methods of nucleic acid sample preparation should be robust. I often prefer analyzing a dilution series of a sample. This permits analysis of linear dilution data to the expected dilution slope. A sample with a slope too far removed from the expected may be problematic for quantitation and should be closely examined before conclusions are drawn. Although real-time PCR is a powerful tool, it still requires a sound understanding of the basics of the technology and the assumptions that are made to draw valid scientific conclusions from the results.

As we move forward in this era of genomic exploration, real-time qPCR will continue to play a central role in this effort. I am certain that continual improvement in instruments, reagents, and techniques will aid this effort. I am happy to have been a part of this story!

REFERENCES

1. Kappes JC, Saag MS, Shaw GM, Hahn BH, Chopra P, Chen S, et al. (1995) Assessment of antiretroviral therapy by plasma viral load testing: standard and ICD HIV-1 p24 antigen and viral RNA (QC-PCR) assays compared. *Journal of Acquired Immune Deficiency Syndromes and Human Retrovirology* **10**: 139–149.
2. Holland PM, Abramson RD, Watson R, Gelfand DH (1991) Detection of specific polymerase chain reaction product by utilizing the 5′–3′ exonuclease activity of Thermus aquaticus DNA polymerase. *Proceedings of the National Academy of Sciences of the United States of America* **88**: 7276–7280.
3. Higuchi R, Fockler C, Dollinger G, Watson R (1993) Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. *Biotechnology (NY)* **11**: 1026–1030.
4. Livak KJ, Flood SJ, Marmaro J, Giusti W, Deetz K (1995) Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. *PCR Methods and Applications* **4**: 357–362.
5. Heid CA, Stevens J, Livak KJ, Williams PM (1996) Real time quantitative PCR. *Genome Research* **6**: 986–994.
6. Gibson UE, Heid CA, Williams PM (1996) A novel method for real time quantitative RT-PCR. *Genome Research* **6**: 995–1001.