Host: This is the podcast for Clinical Chemistry, I'm Bob Barrett.

Rapid advances in molecular diagnostics form the focus of the April issue of the journal *Clinical Chemistry.* Studies using the quantitative polymerase chain reaction are flooding scientific journals as more researchers use this procedure for purposes ranging from pathogen detection to cancer prognosis.

However, some are saying that this deluge of research is hard to interpret or even replicate, because researchers are not using all the same benchmarks.

An archetype consensus guideline for this key laboratory method was published in the April 2009 issue of *Clinical Chemistry*.

Authors of the guideline, including Stephen A. Bustin, a professor at the Institute of Cell and Molecular Science, University of London, and Dr. Carl Wittwer, a pathology professor at the University of Utah Medical School and Director of Flow Cytometry and the Advanced Technology Group at ARUP, recognized that there was a widespread problem in reporting and interpreting quantitative PCR data. Their principles could be considered a researcher's to-do list that details the steps to follow when using quantitative PCR and preparing the data for publication.

Dr. Wittwer, we'll start with you. Tell us, what exactly is polymerase chain reaction, or PCR?

Dr. Carl Wittwer: The polymerase chain reaction is a tool that molecular biologists use to study DNA. It's probably the most important, most basic, fundamental tool that's used today by molecular biologists. It was created around the mid-1980s, and later its creation received the Nobel Prize. It's important because it allows us to look at DNA and RNA, the nucleic acids of life, in a relatively simple way, both for research and diagnostic purposes.

Host: Well, how does this differ from quantitative PCR?

Dr. Carl Wittwer: Ah. Quantitative PCR was a technique that developed as an outgrowth of PCR, specifically for quantifying the amount of nucleic acids. So if PCR was developed in the 1980s, this quantitative PCR was developed in the 1990s, and it essentially incorporated some

automated instrumentation to allow not only detection of a particular target, whether it's an infectious organism or a gene that may be involved in cancer or genetic disorders. It allowed not only detection of those targets but also quantification, and particularly in medicine and some aspects of clinical medicine being able to quantify or count the amount of DNA turned out to be very important.

Host: Now, why is quantitative PCR in the spotlight now? Why is it so important?

Dr. Carl Wittwer: Ah, very good. Quantitative PCR again allows the amount of, say, a viral organism like HIV to be specifically quantified. So if you are in a clinical laboratory interested in following certain disease processes, you may be interested in the viral load — that is, how much virus a patient is currently carrying — and that kind of measurement requires quantitation of the PCR process.

For general molecular biologists, one of the most common uses is measuring transcripts, that's RNA copies. Now RNA, this is kind of messenger RNA. It carries a message from the DNA in the nucleus for coding different genes, and it's carried by the messenger RNA out to the machinery in the cell that actually makes proteins that do all the functions that allow us to live.

So by measuring the amount of that messenger RNA, you can get an idea of how much of this template you are creating. So many different disease processes correlate with differences in these messenger RNA or transcript levels.

For instance, there are lots of different platforms now. You've heard of DNA arrays or chips, and those can give an initial estimate of the amount of message or RNA targets, but the Quantitative PCR is a much more accurate method to do that.

So both for biology research and medicine, quantitative PCR has become more and more important.

Host: Well, exactly how is qPCR performed?

Dr. Carl Wittwer: The techniques developed in the mid-1990s essentially incorporated fluorescence in automated instruments to automatically measure and get estimates for quantification. So the PCR process itself essentially replicates DNA in a test tube. So it

basically does what the cell would do. When a cell divides from one cell to two cells, it has to replicate its DNA to pass it on to its daughter cells. And the PCR takes this process and essentially eliminates the cell. So it's DNA replication in a test tube.

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That process is encouraged and made possible by temperature cycling of the sample, and the quantitative PCR was enabled by observing light or fluorescence from the DNA as it was being amplified. So different fluorescent dyes are added in, and you essentially take a picture or monitor the fluorescence of DNA as it's being amplified. That's called real-time PCR, also quantitative PCR.

Host: Okay. Are there any problems associated with this method?

Dr. Carl Wittwer: The method these days is extraordinarily simple to perform, and that's a good thing; however, over the years, there are many different platforms and methods to analyze the light that comes off, and what we've found over the years is that because it's so simple often many of the technical details are not necessarily revealed in manuscripts and publications, and that makes the results much more confusing and difficult to repeat by other investigators.

So the actual simplicity of PCR and qPCR has been a great thing; but along with that has been a little laxity in reporting how the qPCR reaction has been performed, and that's been a deficit in the literature. That in the April issue of *Clinical Chemistry*, one of the articles tries to address in establishing some criteria and minimal reporting requirements for this very basic reaction in molecular biology as the quantitative PCR method.

Host: Thank you, Dr. Wittwer.

Now, Dr. Bustin, let's go to you. In your opinion, is real-time PCR easier and more reliable to use than standard PCR?

Dr. Stephen A. Bustin: Well, qPCR is easier, more reliable and more informative. It is easier because qPCR results obtain about the need to pull message out. There is no need for multiple opening of numerous tubes, and any interpretation as to whether bounded are actually present or just the smear is no longer necessary. Consequently large amounts of data can be acquired much, faster than is possible in conventional PCR. Reduced hands-on time encourages the use of

replicates and particularly biological replicates. This makes sense, as it allows qPCR studies to be properly powered.

Biological replicates help assess the biological variants and determine the statistical significance of changes in RNA level. This is particularly important when results for just fairly modest changes in mRNA levels; for example, about three or four differences.

qPCR is more reliable, because there is much less physical contamination. Tubes with amplification products need never be opened, thus eliminating a major source of conventional PCR contamination.

And finally, qPCR is more informative, as it has potentially quantitative and thus allowing accurate monitoring of changes in RNA levels.

Host: Well, then, what would you say are the main problems with qPCR?

Dr. Stephen A. Bustin: Well, the relative ease with which it is possible to acquire large amounts of data makes it easy to forget about appropriate measures of quality control, and this relates to both template quality assessment, as well as assay quality assessment. For example, it is essential to know as much as possible about the integrity of RNA, as well as one has to be concerned there is no inhibitor of the RT or PCR reaction present.

And there are various methods that allow researchers to analyze the integrity of the RNA. Unfortunately, what was the patient a few years ago is no longer the patient today. Measuring absorbent ratios or visual inspection of RNA in the gel was adequate when performing northern dot analysis, which essentially gave a qualitative result.

Today, however, conclusions aim to explain small difference in RNA levels, or they might use very, very small amounts of RNA, and all this precludes the use of gels. So we have developed microfluidics-based systems and these are an answer, but by no means the ultimate one, because they give you the result for ribosome RNA and not for messenger RNA, which is what we're interested in.

So there are better ways. In our 2006 *Nature Protocols* publication, we proposed the use of a 3', 5' assay that can measure RNA integrity. Of course,

there are problems with this approach, as well, because, for example, in your supply stains these can interfere with the interpretation of the integrity assay. So the ultimate integrity assay has still not been developed.

The inhibition control is also important, because obviously if a result is due to inhibition, then this gives you a wrong result. And so we published details back in 2006, a very easy inhibition assay we could call the spud assay because it's based on a potato amplicon, and we suggest that people would routinely look at both the integrity and the lack of inhibition in their RNA preparations and DNA preparations for a matter.

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In addition to template controls, assay controls are very important, for the use of no template and no enzyme controls is essential. No template-reaction quality controls for any contamination, whereas the no-RT controls detects genomic DNA contamination.

Negative controls—for example, RNA started on some control patients undetected by a disease or by a pathogen—are also essential.

And sometimes forgotten but also important are positive controls that assure any researcher that the assay is performing as expected.

And finally, appropriate normalization is a real problem with qPCR phase with the use of multiple validated referencing essential for the quantification of modest changes in RNA levels.

Obviously, when one sees tissue culture cells and you identify a very large—for example, a 100-foot change in the on-start of mRNA levels in a single—although it has to be validated referencing, if possible, these are the main problems that I see in qPCR.

Host: Well, do these problems affect the reliability of data reported in the scientific literature?

Dr. Stephen A. Bustin: Yes. That is the problem. Anyone who would take—if you take a brief look at the materials and methods' section of a publication that utilizes qPCR data, one will notice that the majority not provides sufficient experimental detail. This then makes it impossible for the reader to use any experimental detail or even decide whether conclusions are valid.

There are numerous different ways of carrying out even the simplest qPCR experiment. For example, a student that would like to quantify some mRNA from tissue-culture cells treated with a growth factor compares levels of those of control tissues, I could extract total RNA or messenger RNA, but I may or may not assess the integrity and lack of inhibition, as I already mentioned.

I can then reverse them and describe different amounts of RNAs in a wide range of enzymes at different temperatures for different lengths of time and different volume. I then have different protocols that give me different results, and this was published by Mikael Kubista and his team a few years ago. I can use random 6 months, 9 months, 15 months, or combinations of the various planning methods, or I can use gene-specific priming, all of which give me different results. I could do a one-enzyme/one-tube, two-enzyme/one-tube, or two-enzyme/two-tube RT qPCR assay with a wide range of controls or without appropriate controls, and the problem is that it's very rare that you find a paper that actually lists precisely what the individuals have done to get their results.

Furthermore, data analysis is highly subjective and the choice or number of reference genes are not standardized, and reporting of data is also widely divergent. Copy numbers are quoted of full changes; calibrators may or may not be included; standard curves' method in one, the efficiency of the assay may have been assessed, and so on.

And so it's clear that this umbrella term "qPCR" shelters a huge divergence of protocols, and a reviewer or reader cannot easily determine whether the experiments were carried out satisfactorily unless one addresses these issues.

Host: Scientific literature is peer-reviewed, so why aren't technical problems spotted before publication?

Dr. Stephen A. Bustin: Well, yes, that is a very good question. And I think one of the reasons is that reviewers cannot be expected to be expert in every technology that underpin a particular paper. So, for example, they may not know, because they're not expert in that particular technology, what particular issues to look for and what telltale signs there might be that advocate caution.

For example, my expertise lies in nuclear-acid quantification, and if I read a method that describes,

for example, facts analysis it might not be clear to me if a description that's in this materials and methods' section contains a mistake that might invalidate any subsequent analysis of the selected cell population. That's a real problem, because the techniques we use are very specialized, and we can't all be expert in every technique.

In addition, of course, qPCR is generally regarded as a very easy technique, and it's often dismissed with a single sentence of the first protocol published in a previous paper. And then you go back to that paper, and then there's another reference of previous paper, and so on. And this then often means that information and with respect to assay efficiency of primer, for example, is difficult to locate. For example, if a paper was published five or six years, ago it may be difficult to locate that online. Sometimes papers are not open access, and sometimes the information is just not available even in those older papers.

So there is a number of reasons why even though a paper is peer-reviewed, they will still not give you the information that you require to be able to assess whether an experiment has been carried out in a satisfactory manner.

Host: Has this caused any real-life problems? For example, have any oversights caused adverse effects on public health?

Dr. Stephen A. Bustin: Yes, and it's this that has finally galvanized us to go public with our recommendations for guidelines, having talked about them for quite a long time. One of the critical applications of qPCR is its use as a specific and sensitive diagnostic assay for the detection of pathogens.

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So a publication in 2002 used TaqMan technology to report the presence of RNA-only measles virus in a testing of autistic children. And these results were used to support speculation of a possible association between intestinal abnormalities in children with developmental disorders and immunization against measles, mumps, and rubella, the so-called “MMR vaccine.” And the paper claimed that a vast majority of patients with bowel abnormality were positive for measles virus in contrast to controls that were not. And so it concluded based on the qPCR data that there was an association between the presence of measles virus in gut pathology in children with autism.

And at this point, there had already been a huge drop in the number of children being vaccinated in the UK with the MMR vaccine based on the original speculation by Andrew Wakefield when the *Lancet* paper came out in 1998, and this drop in vaccination has led to several deaths both in the UK and in Ireland. Certainly it's been a real public-health problem here, and vaccination levels are only slowly returning to the levels recorded before this gaffe. And indeed a recent report suggests that measles virus is now endemic in the UK.

And so in 2007, there was the first of about 4,000, I mean, 5,000 cases in the United States on the autism proceedings came to trial at the U.S. Court of Federal Claims in Washington, D.C. These trials were designed to establish whether or not autism can be caused, amongst other things, by MMR vaccination.

Now, I had previously reviewed virtually all the data underlying the 2002 paper for reports filed by the three principal defendants in the UK in MMR litigation, and I was also asked as an expert witness for the Washington proceeding. And my examination of the data, the raw data from the actual paper, they revealed the catalogue of technical problems that included unclear data reporting, inappropriate data analysis, non-reproducibility assay, and evidence of widespread DNA contamination. And so although virtually every problem on its own was terminal for the conclusions offered in the paper, none of this was apparent from a simple reading of the paper.

Incidentally, the verdicts in the three cases that came to trial have just been announced, and every case of verdict went against the claimed association.

And so this highlights a tremendous hazard that's inherent in interpreting results obtained using such a superficially simple and sensitive yet potentially easily contaminated assay, and the lesson really is that we cannot afford to allow substandard publications to contaminate the scientific literature.

Host: Well, with this in mind, what's the practical solution to the problem?

Dr. Stephen A. Bustin: Obviously, there are different solutions. Now, we believe that one of the solutions is transparency. And so what we feel is that if all such follow the checklist that describes the essential parameters that then allow an outside reviewer or reader to evaluate the

likely validity of a result, and if the journal acknowledges this and includes the details in the publication or as an open-access online supplement, then the reader can rest assured that the technical problems associated with the publication have been addressed, and they do not have to worry about the technical problems any longer but concentrate on the biological relevance of the data presented.

And so we feel that these mighty guidelines that we have proposed would go a long way toward establishing reliable standards and increase people's trust in the validity of results presented in the papers that underlie qPCR data.

And eventually, of course, it will be great to speak with one voice, and this is where the use of common language for data export comes in. And it's called "RDML," this common language, and it is the brainchild of a group of Belgian scientists led by Dr. Hellemans and Professor Vandesompele from Ghent, and this promises them to allow anyone to reanalyze anyone else's data. For example, if I have a collaborator, if he sends me his results using this RDML language, I can then input it into my program and actually reanalyze the data using my parameters. And really what it does is, it increases the confidence in the results that are being published.

Host: Thank you, Dr. Bustin.

Our guests for this podcast from the journal *Clinical Chemistry* have been Dr. Steven A. Bustin, professor of Cell and Molecular Science at the University of London, and Dr. Carl Wittwer, professor of pathology at the University of Utah Medical School.

Host: I'm Bob Barrett. Thank you for listening.

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