The Future of qPCR Best Practices, Standardization, and the MIQE Guidelines

[0:00:00]

Sean Sanders: Hello and welcome to this *Science*/AAAS webinar. My name is Sean Sanders and I'm the commercial editor and webinar editor at Science.

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In today's webinar, we are delving into the somewhat murky depths of quantitative PCR and the so-called Minimum Information for Publication of Quantitative Real-Time PCR Experiments or MIQE guidelines.

Quantitative PCR has emerged as a powerful tool in molecular biology laboratories, both in research and in diagnostic settings. Even as qPCR grows in popularity, it is being recognized that there are some challenges associated with the technology, particularly with respect to reproducibility within and between laboratories. Fortunately, many of these limitations can be addressed with a standardized set of best practices. Using the recently published MIQE guidelines as a foundation, our expert panel will address the best practices of qPCR with the goal of providing researchers with more consistent and reliable data.

With me in the studio today I have three expert scientists to help us understand this particular subject. Just to my left is Dr. Stephen Bustin from Barts and the London School of Medicine and Dentistry in the United Kingdom. Next to him is Dr. Greg Shipley from the University of Texas Health Science Center in Houston, Texas. And finally, we have Ms. Manju Sethi from ThermoFisher Scientific in Wilmington, Delaware. Many thanks to you all for being with us today.

Each of our speakers is going to give a short presentation, after which we will have a Q&A session during which the panel will address the questions submitted by you, the live audience.

A reminder to online viewers that you can see an enlarged version of the slides by clicking the enlarge slides button located underneath the slide window of your web console. You can also download a PDF copy of the slides by using the download slides button. As I mentioned, if you're joining us live, you can submit a question to the panel at any time by typing it into the ask-a-question box on the bottom left of your viewing console below the video screen and clicking the submit button. Please do

remember to keep your questions as concise as possible as this will give them the best the chance of being put to our panel.

Finally, thank you to Thermo Scientific NanoDrop and Solaris Products for their sponsorship of today's webinar.

Now, I'd like to introduce our first speaker for this webinar, Dr. Stephen Bustin.

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Dr. Bustin obtained his Ph.D. from Trinity College in Dublin and is currently professor of Molecular Science at Barts and the London School of Medicine and Dentistry, a part of Queen Mary, University of London, and serves as visiting professor of Molecular Biology at the University of Middlesex. His research group's general areas of interest are small and large bowel, as well as colorectal cancer with particular emphasis on investigating the process of invasion and metastasis. Dr. Bustin also has a special interest in real-time PCR and has written and edited two books and published numerous peer-reviewed publications and book chapters on this subject. He coordinated the recent MIQE initiative.

Dr. Bustin, thanks for being with us.

Dr. Stephen Bustin: Thank you very much, Sean.

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There are several reasons why real-time PCR is very popular and these are three of those. People believe it is a simple technique, people believe it is a mature technique, and it is very often referred to as the gold standard.

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The problem is that when you delve a little bit deeper, it's none of these things. Typically, real-time PCR is actually very complicated because there are numerous steps involved in getting the assay to work. So it is not simple. It is complex.

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In addition, it is not a mature technology because it is evolving. New chemistries, new reagents, new algorithms are being developed all the

time and they change the way we carry out our real-time PCR experiments. And finally, it is not a gold standard because the results we get are very variable.

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Let me illustrate this too using this particular paper which was published a couple of years ago, a very important paper. It's a diagnostic paper that compares real-time PCR assays that are used in clinical diagnostics, so very important issue. And of course, if we go to a hospital, we want to be certain that the results that are carried out or the tests that are carried out are accurate and reliable.

Now, this paper concludes that there's considerable variation in the performance of clinical real-time PCR assays. What does considerable variation mean?

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Well, if you look at the results and I've blown them up for you here, these different assays have massive differences in the delta-Cqs between the best and the poorest assays. So it means that if you go to a particular hospital to have an assay done on you and the assay is not cut out terribly well, you will not be diagnosed appropriately and you will have consequences and these are sort of consequences for your health obviously. So there is clearly considerable variation in diagnostic clinical assays from assays that people are running, and this is a problem.

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The key issues, there are numerous key issues, but these I think are the main issues. The sample quality, that is incredibly important. If I bake a cake, I want to use the best quality ingredients and I want to be certain that my eggs and butter are fresh. Why wouldn't I be able to do the same for real-time PCR assay?

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It has been shown on numerous occasions that the way you do reverse transcription is very important and it gives you a different result if you do things inappropriately. Assay validation optimization. You wouldn't dream of running a marathon without optimizing your performance, so why would you do that for important diagnostic or a research assay? PCR efficiency we know is an exponential amplification. So if PCR efficiency

varies or we know nothing at all about it, then we may well have problems with interpreting our data.

And perhaps the most important issue or certainly amongst the most important issues is normalization. If you're driving a car in a 30-mile zone next to a field with sheep and the police stopped you and complained or charged you with speeding, you would not be terribly reassured to hear them having measured your speed relative to the sheep that were running up and down next to you on the highway. So why would you be willing to do your normalization using reference genes that you know very little about and you certainly have not validated?

So there are a number of key issues that are already common sensical, and if you think about these or if we think about these, there's no reason to believe that we shouldn't take care in how we carry out our experiments, and what MIQE does is it takes these common sense parameters and puts them into an easily accessible and easy-to-follow set of guidelines.

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This is the kind of paper that's being published.

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In this case, some of the information is provided and they look at the efficiency of the PCR reaction, looking at relative quantification and comparing it -- amplification efficiency is not totally impossible to achieve. So you can be fairly certain that whatever conclusions this paper comes to, it is probably not going to be terribly accurate.

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Now, you might imagine that this is confined to second-tier journals, but I have analyzed the real-time PCR papers published in top-tier journals and here's a particular example I want to show because it was of real interest to me. Because this paper was published in June of this year in Nature and it claims a functional role for pseudogenes, for RNA produced by pseudogenes. It coins the term competitive endogenous RNA and the purpose of this paper is to show that pseudogenes produce RNA that binds to the mRNA of actual genes and regulates that gene expression. So very, very important paper based on real-time PCR.

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Now, if you look at the real-time PCR data, the first thing you'll note is that these are marginal results. For anyone who does real-time PCR, you can see a 50-fold or a half-fold change, 1.2-fold change, 1.5-fold change, so very, very small changes. I'm not saying that they're not real. What I'm saying is if I'm looking at this paper, I would like to understand how the authors actually went about doing the real-time PCR assays. So unfortunately, most of the time, papers don't publish their methods anymore. They go online for full methods as does Nature.

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This is their methods summary online. As you can see, they mentioned real-time PCR, but that's the only information that's provided on how they get the assay. So you have no idea how they came to their conclusions.

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They do provide some pieces of information. They provide the primer sequences. And if you look at the primer sequences, you can see that there is a mismatch at the 5' end. It probably doesn't matter but it tells you that even in Nature, you can get papers published where the primer sequence is incorrect.

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Similarly, they then show the reference sequences of the genes they used.

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However, if you look at the reference sequences, then what you find is they provide the wrong reference sequence because it's an NM sequence they published when it should have been NR sequence for a pseudogene. So small mistakes like that are important, and the point here is that at least we have that information. What usually happens is you don't get that information because it was not published in the papers.

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The end result is two-fold. As a researcher, what happens to me is I have to retract my results and that is of course not very good.

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Here is a paper published in 2005 in Science and it'd have to be retracted as the retraction says, "Because the real-time PCR data were analyzed incorrectly." So it's a blow to our ego.

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More importantly, if we're using diagnostics, this paper was published in 2002 and it purported to confirm an association between measles virus and gut pathology in children with developmental disorders such as autism.

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The consequence of this paper was that today, measles is endemic in the United Kingdom, and that in parts of London, fewer than 50% of children are vaccinated. This was part of a court case in the United States in Washington D.C. three years ago and I was an expert witness of that, where I was able to show that the data underlying these were not terribly appropriate. So a paper, a diagnostic paper uses real-time PCR data and has incredible consequences for people's lives.

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Key issues then as I said, sample quality. What I did was here, I looked at every single real-time PCR paper published this year in Science, and a lot of them published in Cell and the BMC stable of journals, and looked at the key issues that I told you earlier on the left hand that I think are important. As you can see, not a single Science or Cell paper looks at sample quality and it's the same for Nature. Notice a very few of them look at the RT or give you information about RT. Not a single Science paper optimized the assay. PCR efficiency was not optimized or was not looked at in most papers, both Cell and Science. Normalization: every single Science paper normalized against a single reference gene and none of which was validated.

As you can see, BMC journals have a much better track record of publishing papers and data that are technically likely to be more correct. The problem with data analysis is, and that's where normalization comes in, that it acts as a gatekeeper for how we present our data, and that's why I think it's so important to have programs such as a qbasePLUS for

example that actually do the analysis and make sure that both the data handling the statistic analysis are correct.

And so in summary, all I'd like to say is if you look at the literature, you find that virtually every paper that is published doesn't give you the information required; and very often, if the information is provided, the information is incorrect, and that's what MIQE is trying to address.

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Sean Sanders: Great. Thank you very much, Dr. Bustin.

Our second speaker today is Dr. Greg Shipley.

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Dr. Shipley completed both his undergrad and graduate training at the University of California, Santa Barbara, after which he moved to University of Florida in Gainesville and then to MIT in Cambridge, Massachusetts to carry out his postdoctoral training. Following academic appointments at Texas A&M University and the University of Houston in Texas, Dr. Shipley moved to the University of Texas Health Science Center in Houston Medical School in 1990, where he is currently the director of the Quantitative Genomics Core Laboratory. Dr. Shipley oversees the running and analysis of all real-time qPCR assays as well as the development of new assays. He is also one of the authors of the MIQE guidelines.

Dr. Shipley, welcome.

Dr. Gregory Shipley: Thank you very much.

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Yes. What I want to talk to you today about is three topics. The first one is what is real-time qPCR because not everyone watching today might actually know about this technology. Then I want to talk about two issues, two sections of the MIQE guidelines and try to smooth your way to satisfying those requirements.

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So first of all, what is real-time qPCR? I have previously made my own definition here, which is collecting an increasing fluorescent signal for

one or more gene targets in real time during a polymerase chain reaction or PCR, leading to a quantitative value for each target. And what I want to show you now are the two most basic ways of signaling in a real-time qPCR reaction, and the first is based on hydrolysis probes. They rely on a central oligo in the center with the R and the Q which you can see there, and that is the probe.

The probe is quenched by a phenomenon known as FRET or fluorescent resonance energy transfer or Förster resonance energy transfer depending on which way you want to define that. And in this probe, as long as the reporter and the quencher are in close proximity to one another, the signal from the reporter will be quenched. As the reaction moves on as you can see in the bottom panel there, taq polymerase, as it also polymerases, will displace and cleave the reporter; and therefore, as the proximity to the quencher is removed, the reporter will give off a fluorescent signal. So in every assay, three things have to be working in concert: the forward primer, the reverse primer, and the probe.

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The second most common method of signal generation in real-time qPCR assay is based on SYBR Green I. In this case, a soluble dye, SYBR Green, gives nearly no fluorescence when it's in a free solution. However, as the polymerization occurs and the double-stranded molecules are formed through the PCR, the dye binds among a group of DNA and it increases its fluorescence over a thousand-fold, thus giving an increasing signal as the polymerase chain reaction proceeds.

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So what does that data look like then? So in the center panel here, you see an amplification curve. This is based on a hydrolysis probe. It's from the human PTEN assay and this is the 7-log standard curve, which is something that I'll be showing you over and over during this talk. It's not that hard to generate this. What you can see to the left are the values that come out of this. Normally, there's a threshold on that amplification curve for a lot of software. The Roche instrument does not require that to get a value; but nonetheless, these are how many cycles. So the 12.65 you see in the table to the left is how many cycles it took to get a measurable signal on this instrument for that very first standard point and on down the line.

[0:15:07]

What you see down below that is the result of that. This is the standard curve itself which can be drawn by the software, and to the right you see the statistics for this assay, and I will be going over those later on in more detail.

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So first of all, PCR target information.

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So these are the requirements through the MIQE guidelines for PCR target information. The things marked in E are deemed essential and should be in every methods section. The D is desired and we'd like to see them, but if you don't have them, it's not as critical. As you can see, gene symbol, the accession number, amplicon length, all of these things that are marked in E are important.

Secondary structure is something that's very important when you are designing an assay because you don't want to put your primers on top of a large stem structure, and that's something I don't have time to go into more right now, but I did put the reference for the m-fold server there and you can put your sequence in there and get a fold at that site.

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So this is what the header from a RefSeq gene for transcript, an NM. As you can see, there's the NM there under accession number. At the top I've put three useful websites. First is the NCBI which is at the NIH itself. It's a very valuable database. It is in fact the homepage for my web browser just to show you how much stock I've put in this one. The UCSC site at UC-Santa Cruz is also an excellent site particularly for DNA and genomic data. And then the EMBL site is also excellent for folks who are in Europe or not in the US.

So the accession number then is represented by NM underscore with usually six but perhaps more digits as more and more of them are known. This is for a transcript. The letters would be different if it was for a DNA sequence or as Stephen pointed out, for a pseudogene for example.

The actual abbreviation gene symbol for this gene is Slc26a4, which stands for the soluble carrier family 26 member 4; however, the investigator who asked me to make this assay calls it Pendrin, what you can see is under the common name there. And the problem with

common name of course like as in plants is we all know the local common name, but it becomes multiple different species as you get out into the real world.

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So how about getting the template specificity issue? Well, at least in silico, you can answer that question by blasting your PCR amplicon sequence against the database, and as I pointed out up here, I use BLASTN rather than MegaBLAST because it's actually more -- it's not so sensitive I guess is what I put there. What I mean is it will pick up many more sequences that do not exactly have the primer sequence. You want to look for anything that might even have a chance of interacting with your amplicon. As you can see in this case, this particular one has a single hit, which is exactly what I was looking for.

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Another issue is how do we know about exon-intron boundaries? Well, if you look at RefSeq sequences from mouse and from human, if you look in the RefSeq sequence, you'll find in the header, you'll find this information. However for the rat, because this has only been more recently sequenced, you can't find this information. However, the splice junctions for the higher mammalian species are highly conserved.

So in this case, what I've done is I've put in red as you can see for the rat sequence, 911 represents that C in the CAGT as the 5' base, the same with the 988 and the same with the 940, and I'll go over this later on in the probe. So in human, 911 actually corresponds to base 1114, because there are different lengths in the sequence, and 1191. And if we look at the data down below, you'll see that 1114 falls within exon 7 and 1191 falls within the human sequence exon 8; therefore, we know even in the rat that this is going to cross the 7, 8 exon boundary in the rat most likely.

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So here is that table. This is information for a table. This is not exactly how you would express it perhaps in your publication, but this is all of the data that you should have there. First of course is the species, the name of the assay. You might give some synonyms. That would be helpful. The accession number is essential. Again, as I've pointed out, 911 is that first C base, 988 is the A, 940 is the A on the probe. You can see that this has FAM as a reporter and the BHQ, the black hole quencher, is the quencher on this probe.

And you notice on the probe, I also have a plus there. What that tells you is that this probe binds to the same sequence as the forward primer. There are minus probes I don't have plenty in my database that would bind to the same sequences in reverse. So it's just a natural piece of information that's good to report. 78 base pair PCR amplicon length, you can get that by subtracting 988 from 11 and adding 1; crosses the 7/8 boundaries we'd just shown. The PCR efficiency is 97% and I'll tell you more about that later; and the low level of detection, limit of detection, is 23 copies. You can also report this as a Cq value. You don't have to use copy number. It just depends on what you're doing.

[0:20:08]

There are no known splice variants. I got that again from the RefSeq sequence. There's the single target by BLAST. I showed you how we got that. There are no folding issues by m-fold and I did that at the m-fold server just as you can.

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Okay, so qPCR assay validation then.

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So here is another list and another list of requirements: desired and essential. First is specificity. This is critical for your assay. If your assay is not specific to what you're looking at, then your data are suspect to say the least. For SYBR Green I, they want you to report the Cq of the no-template control. That's because primer dimers can be somewhat of an issue there more than for a probe beta assay. Calibration curves, I'll show you those; how do you calculate the efficiency, r^2 linear dynamic range or the limit of detection; Cq variation there; evidence for the LOD; and we're not going to talk at all about multiplexing today.

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So if we talk about hydrolysis probe qPCR validation then, this is a probe-based assay. You can see that it looks fairly nice. I made it myself. Thank you very much. It's human IAPP assay. It uses a single-stranded oligo. I purchased that with every one of my assays because we run standard curves with every assay. But that is not required. It is something that we do in our core lab as a service to our customers.

The Cq values in this assay vary from 13. Because the cycle number is lowest where the highest amount of template is, the number of cycles it takes to get there, this curve has a negative slope as you can see and it goes all the way down to 33. So although I am reporting a range of 10 million to 10 copies as in the linear range for this assay, you could also report the limit of detection for this assay as 33 cycles. That would be perfectly legitimate. And then when you do your delta-delta Cq or Ct or whatever you want calculation, you keep in mind that if an unknown has a value higher than that, then you probably should not use it in your calculation, and that is the key to the whole thing here.

Assay stats: The slope is -3.435. The r^2 is .999. The y-intercept is 38 cycles which is pretty normal in my experience. PCR efficiency is 95.5% and the way to calculate that, the little formula is right there on the screen. Variation at the LOD as you can see is not significant. The minimum QC requirements for our core lab is that the assay has to have a qPCR efficiency of 93 or greater, which is a slope of -3.5, and it has to go less than 30 copies in order to pass QC validation.

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So what if you're using a SYBR Green assay, which most of you are? You really have to show something about template specificity. The two I don't say easiest, but the two ways to do that or probably the best one is asymmetric restriction digest and showing that on an acrylamide gel. At least run it. You don't have to show it in your methods section, but you have to say, "We did this experiment. We got a single band. We cut it with this one restriction enzyme. We got the two band sizes that we expected, and that's pretty much all we saw on the gel." Or you can simply have the thing sequenced. However, if you do have multiple bands, your sequence isn't going to look very good. So that's the downside of just sending it out for sequencing.

You want to make sure you're discriminating among multiple sequences. If you have closely related family members, it can be tough with the primer-based assay. A melt curve alone is really not sufficient to show template specificity, but it is incredibly useful information and I highly encourage everyone to do so. You see the assay on the bottom left has some fairly tight peak corners. The one on the left might have a slight shoulder to it. I'm really not a SYBR Green aficionado so there could be something else lurking under that melt curve. You really need to run a gel and find out.

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So what if you want to validate using a SYBR assay? You didn't buy a standard or you bought it from a company that you don't know the sequences of the primers and you don't have any way to get that information. How can you validate your assay? It's really very simple and I'll outline it here on my last slide.

So originally, you're going to run this in either an RT-PCR or a PCR reaction. That is your first run. You monitor that run with real-time qPCR and you get your Cq value. Depending on what that is, so for example, if the Cq value is 30, then you want to dilute perhaps only 1 to 100 or 1 to 1000, okay? If it's 25 or higher, you definitely want to start your dilution around 1 to 10,000. A couple of 100-fold dilutions are very quick to do. And then from there start making a 7 or 8-log standard curve.

It's critical that you dilute this in E. coli tRNA or linear acrylamide or some carrier. Otherwise, you're going to have a real problem. Things will be sticking to the plastic and it will be not so good. Run your dilution in duplicates in a PCR as you see in the figure for this Rhesus monkey collagen 1A1 assay. Collect the data and make a standard curve in the software and then you have your stats.

[0:25:05]

So how did this one work out? Well, of course I'm showing it 'cause it worked out really well. The slope is -3.12 for this particular assay. R^2 value is very tight. The y-intercept is 36.7, which is again normal. PCR efficiency, gosh, it's 100%. Nice study. The LOD for this, however, is 33 cycles. I don't know anything about this. I do know the primer sequences for this, but if there's one I didn't, you know, then this is the only data I would have to tell me what the limit of detection is going to be. So this is it and this is what you have to take to the bank when you're doing your unknown quantification.

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Finally, I just like to show quickly folks in the lab who are important, especially Ms. Xiaoying Wang who is my right-hand person. Sean?

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Sean Sanders: Great. Thanks, Dr. Shipley.

And we're going to move right on to our final speaker for today, and that is Ms. Manju Sethi.

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Ms. Sethi joined ThermoFisher Scientific in 2008, and as senior product manager is responsible for the development and management of its NanoDrop line. Ms. Sethi specializes in the unique technology and workflow solution needs of life science customers and brings over 20 years of biotech and life science product management, as well as marketing and business development experience to her team. Previously, Ms. Sethi was a business consultant for various Fortune 100 companies and was also a senior member of the management team for the Qualicon Biotech Division of DuPont, where she served as the Director of Strategic Planning and Director of Business Development. Ms. Sethi holds a Bachelor's Degree of Technology in Chemical Engineering from the Indian Institute of Technology and a Master's in Chemical Engineering from Clarkson University.

Welcome, Ms. Sethi.

Ms. Manju Sethi: Thank you, Sean, and thanks to Doctors Bustin and Shipley, you now have a good overview of qPCR validation, the MIQE guidelines, and some of the dire consequences of not following standardized best practices. So what I'm going to take you through here today are some practical applications.

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Take some examples. Dive in a little deeper to a couple of examples within the MIQE guidelines; and specifically, I'm going to focus on nucleic acid, the QC of nucleic acid, as well as some of the aspects of qPCR assay selection.

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So the first topic is the QC of nucleic acid. As Dr. Shipley had showed you, there is a checklist in the MIQE guidelines; and within the QC of nucleic acids section, there are three particular topics that I'm going to speak to, which is the quantification of nucleic acid, which is deemed essential, and also the purity assessment and RNA integrity.

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So why are these parameters so important? As you must have gleaned from the previous two talks, if you don't follow best practices, one of which is quantifying your nucleic acid after extraction, what you can end up with is no results or worse yet, erroneous results. Similarly with purity, if you don't know what chemical contaminants you have, you will not be able to assess whether that has an impact on your downstream results or not. And integrity, whether you have chosen to clean your RNA, whether you used DNAs or not, you need the information to know what your starting material is; and therefore, an RNA integrity check is indicated.

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So what are the tools available to do this with? For quantification and purity, UV-Vis spectrophotometry is a universally accepted method, quantification using the absorbance at 260 nanometers and purity assessment using the ratio of 260 to 280.

In addition for quantification, fluorescent RNA-binding dyes can be used. They do require the setting up of standard curves and they do require use of consumables; and usually, fluorescent-based methods are indicated when you have very low target concentrations or you require specificity. If your target concentrations are in a sufficient range, you're able to use the ease of UV-Vis spectrophotometry for both quantification and purity assessment. Some of the technologies mentioned in the MIQE guidelines include the Thermo Scientific NanoDrop spectrophotometer as well as RiboGreen.

For integrity, there are a number of methods including standard gel analysis and instruments that provide you with integrity numbers or quality indicators, and these are also included in the guidelines.

We had indicated that you could get erroneous or no results if you fail to do your quantification properly.

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These are some quick examples of a standard curve in absolute quantification, whereby if you extrapolate you could get the effects of reduced efficiency or contamination or background effects that give you incorrect answers.

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Similarly, in gene expression, if you use normal template quantity as you see with the chart on your left, you're going to get very tight results, very interpretable results, confident results, whereas if you use low template quantity as is indicated by the chart on the right, you're going to get a great deal of variability.

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Another application for qPCR being SNP genotyping, the chart on the left will show you when you use normal or optimal template quantity that you can make good allele calls. The chart on the right with low template quantity yields no allele calls and sometimes it can read to erroneous allele calls.

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So a lot of times you've heard of the normalized gene usage. We've had Doctors Bustin and Shipley talk about it. Are you using one normalization gene, multiple? When you are using normalization genes, the validation of those genes is critical, and while you're doing the validation of those genes, you need to take RNA from multiple samples, equalize the template loading, and for all those, these QC practices still pertain.

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Now, let's move on to purity. Purity assessment of nucleic acids is a little understood factor, especially the assessment of chemical contamination. What is commonly known and understood is that the 260/280 ratio gives you an indication of purity with respect to protein contamination.

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What I'm going to talk about a little bit is the 260/230 ratio and the importance of spectral data which is very rich in information, and by looking at it through your UV-Vis spectrophotometer, data can glean a lot of information about chemical contamination.

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Some examples of contaminants are glycogen which is frequently used to precipitate DNA.

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EDTA which is a chelating agent. In all of these examples, you will see that they have absorbent profiles and significant absorbance at 230 nanometers which could affect your results and your ratio, 260/230 ratio.

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Again, TE buffer has absorbance at 230.

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And I'm going to take you in detail through an example of phenol contamination. Phenol's absorbance profile looks like this where you see a peak at the 230, the 270, as well as contribution in the 260.

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So what can that do to your sample? In this example, the spectra designated as A is pure DNA, not contaminated. The spectra designated as B is contaminated with phenol. And as you can see, the 260/230 ratio gets affected and the peak is shifted, a great clue for you that you have some contamination there.

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Guanidine is typically present when you use extraction kits that's used in the wash step.

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And what can that do to your sample? Again, A is the uncontaminated sample spectrum and B is the one that's contaminated with guanidine. It's going to significantly lower your 260/230 ratio.

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So really, the spectra are talking to you. Listen to it. It will give you wealth of information and you can make the determination on whether those contaminants can be tolerated in your downstream processing. For integrity as I said, gel electrophoresis methods are indicated.

So with QC in summary -- use of complementary methods. If UV-Vis spectrophotometry is appropriate, it can give you quantification. As well

as the purity information, complement that with an RNA integrity technique.

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Now moving on to qPCR assays. We've heard some discussion today about SYBR-based detection or probe-based detection. Dr. Shipley had gone into that with some detail.

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Just at a very high level, when you're making the decision on which method to pursue, here are some considerations. SYBR-based detection is commonly indicated and recommended if it's a high throughput screening application. SYBR-based detection does not allow you to multiplex and it does, as Dr. Shipley indicated, bind to any double-stranded DNA formed.

On the other hand, probe-based detection may be indicated for a more focused approach if you want your assay to bind selectively to the target and it can perform multiplexing.

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So there's so many choices out there which assay to choose. Do I use multiple assays to address all the known splice variants with my gene of interest? There are many considerations.

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But a very important one that is identified in the MIQE guidelines is the publishing of the primer and probe sequences. This again is in that very useful checklist; and as you can see, the primer sequences is listed as essential, and the probe sequences, the requirement for that to be published is listed as desirable. But mind you, there is a little footnote with that desirable and the authors do go on to say that the disclosure of probe sequences is highly desirable. The only reason they hadn't put it as essential is because not all vendors do disclose that information. And as is indicated in red, the authors do say use of such assays is discouraged.

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So what are some of the performance criteria? These were addressed in Dr. Shipley's talk under qPCR validation so I will just hit on them very quickly. When you're looking at assays to consider, consider the efficacy of amplification, the dynamic range, the limit of detection, certainly whether you need to use multiple assays to cover all the known splice variants for your gene of interest, and of course specificity. But overriding through all of this is are the primer and probe sequences published? Are you going to be credible with your results?

Examples of a very MIQE-friendly assay is the Thermo Scientific Solaris qPCR gene expression assays. These do actually cover all known splice variants of genes of interest and are published sequences for primers and probes, and they also have the RNA Spike Control Kit.

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So in summary, the MIQE guidelines is something that we certainly at ThermoFisher Scientific highly endorse. Use that checklist, I know we do, as you design and conduct your experiments, and selecting the right tools and assays before you begin is essential for you to have confidence.

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Sean Sanders: Great. Thanks very much, Ms. Sethi, and thank you all for the excellent and very enlightening presentations. And we are now going to move on to the question-and-answer session to look at some of the questions submitted by our viewers. Just a quick reminder to those of you watching live that you can still submit questions by just typing them into the ask-a-question box and clicking the submit button.

So the first question I'm very quickly going to put out there, I don't think it was mentioned specifically in any of your talks. Where are the MIQE guidelines published? Where can they be found? I'm assuming Google would be a good start, but maybe there's a site you can point people to.

Dr. Stephen Bustin: Clinical Chemistry.

Sean Sanders: Clinical Chemistry.

Dr. Stephen Bustin: The Journal of Clinical Chemistry had published them.

Dr. Gregory Shipley: It's I think 2009.

Sean Sanders: 2009 in the Journal of Clinical Chemistry.

Dr. Gregory Shipley: It's free access.

Sean Sanders: Okay, excellent. So there you go; a good place to start.

So we've had a lot of questions that have come in and we have limited time to get to them so I'm going to jump right in. Here's a question. If I don't intend to publish my work, is it still important to follow these guidelines? I think a bit of a leading question, but Dr. Shipley, do you want to start us off?

Dr. Gregory Shipley: Sure, why not. Well obviously, following the MIQE guidelines is a guideline, and we put them out there so that people would, you know, have a way to put together their experiment in a way to get credible data. And so it doesn't really matter whether you're planning to publish it or whether you're working for a company as this top, top secret information. If the data is not credible, then you're just wasting your time.

Sean Sanders: Okay. Dr. Bustin?

Dr. Stephen Bustin: I guess the only thing I would add to it is that we actually say in the paper that it's for designing and reporting. So it gives a nice checklist, a blueprint for anyone who would like to design an assay. You know if you follow these guidelines, your assay will be a good assay or should be a good assay. We can't guarantee it of course.

Sean Sanders: Ms. Sethi, something to add?

Ms. Manju Sethi: Certainly, our NanoDrop spectrophotometers and Solaris assays are being used in qPCR labs that don't publish. They have other applications and we strongly recommend to them that they follow these best practices.

Sean Sanders: Okay, great. So I'm going to ask you a couple of more technical questions that I've got in that I'm going to try to broaden the discussion a little bit and come back to the MIQE guidelines as well. I've had a number of question come in with a very, very basic issue and that's contamination. So this question in particular asks say that there is contamination in my no-template controls. What are the key points in a protocol I should be considering to avoid this? Dr. Shipley?

[0:40:02]

Dr. Gregory Shipley: Well, PCR in general is fraught with potential for contamination, and so similarly, as you would set up perhaps a special bench to perform RNA isolation and working with RNA in general and then keeping those reagents separate, when you work with the result of the PCR reaction, we have a special room where our instruments are. That's our dirty room. And so if we're going to take, as I pointed out in my talk, if we're going to take some of the PCR product and use it for a standard curve, we will make that 10,000-fold dilution in that room, you know. That plate will never be opened and brought back in to the main lab because it is fraught with contamination.

One place people don't often look is in their water for example. And I would also just quickly say, don't look for where exactly it is. It doesn't matter if it's in one primer or the other or your probe or in your reagents. Just dump the whole thing and start over again, because honestly, you can spend weeks trying to figure out, and at the end it's not very satisfying.

Sean Sanders: Uh-hum, uh-hum. Dr. Bustin?

Dr. Stephen Bustin: Well, one thing I would add is that what we do is when we get a set of primers in from manufacturers, before we do anything, we would simply do a PCR reaction. No template and nothing just to make sure that the primer coming from the manufacturer is not already contaminated 'cause we have found that. That's a very good thing to do. You first try to get a primer set just to see if they amplified anything. If it does, complain. If it doesn't, then what we do is we aliquot our primers and our probes and always have samples that we know are good. At least then we can go back to a set of primers that's going to not be contaminated. And as Greg said, if there's contamination, well, you could spend months trying to find the source. You just throw things out and start again.

Dr. Gregory Shipley: It is important to make working stocks. We have 20 micromolar working stocks and then we have 100 micromolar stock stocks if you will. The -80, you know, that's our archive. And so if the -20 aliquot gets messed up, then you just toss it and start and you go make a fresh one. But make sure when you make the fresh one that, you know, you're not just going to add in the same.

And the other thing people don't seem to get the concept is you can have reagents that are contaminated for one assay. Those reagents will work like gangbusters for everything else but it's only that one thing that's in there. They think if it's contaminated at all, it's forever gone. Well, that's not necessarily true. Surely the primers and prober are gone, but the

reagent, it might be your master mix. If you have it contaminated for one assay, you can use it for all the other assays. If you can't discriminate A from B then we're out of business. So it doesn't matter that it's contaminated for one as long as you don't use it for that one.

Dr. Stephen Bustin: It's probably also worth mentioning that it depends what the level of contamination is. If you have an assay that is detecting abundant target and your Cqs are at 15, 16, or 20, it doesn't really matter if you've got something come up at 35. Obviously, it does matter when you're looking at 30 and it's 35.

Sean Sanders: Okay.

Dr. Gregory Shipley: Yeah, yeah.

Sean Sanders: Great. I've got a question asking about trying to decide between Taqman and SYBR Green technologies, you know. If you were trying to decide, where will you start? What are the criteria you should be considering? Dr. Shipley, you look keen to jump in.

Dr. Gregory Shipley: Well, the thing is that amongst those of us who are the authors, you can probably almost -- I don't know down the middle but you can divide us into two camps. There are those who are violent SYBR Green defenders and those like myself who are on the probe side.

Sean Sanders: Okay.

Dr. Gregory Shipley: I think it depends on -- it depends somewhat on your target template like the one I showed that had no splice variants and had no relatives around. You know that SYBR is really easy if you're trying to -- I would find it very hard to get in and find a family member that had one or two really closely related sequences from related family members and try to parse that out without using a probe-based assay. I would find that very difficult.

A lot of times just for example of the economics and people go, "Well, I'd buy SYBR one time. I'd have to buy it again." Well, you can make your own master mix, but that SYBR Green master mix costs more than the probe-based master mix. And if you say, "Well, I have to buy the probe one time. Now I'm done with that. And now, I don't have to pay so much every time I run the assay." So the economics are not as simple as people think they are.

Sean Sanders: Right.

Dr. Stephen Bustin: Yeah. We use both assays and we would generally design an assay from scratch so we can have a possibility of having a probe. So we would use either say Biosearch online program or we use Beacon Designer, and so we design our assay and usually, we'd use SYBR Green. But if you have a very local pin of a target or if you have problems with primer dimers or as you say if there's other issues, then we would go to the probe, but in general, we tend to use SYBR Green.

Dr. Gregory Shipley: Tee. Very split on the panel.

Sean Sanders: Do you want to be the deciding word?

Ms. Manju Sethi: No. I'd say it depends on the end application, exactly as they were saying. So there's every reason to believe that a lab will have use for both.

[0:45:00]

Sean Sanders: Great. So coming back to the MIQE guidelines for a minute, how do the MIQE guidelines apply to commercially available primer probe sets and do you need to establish the efficiency of the set if you are doing relative quantification. So maybe we'll start with Dr. Sethi on this, talking about whether the MIQE guidelines apply to commercially available probe primer sets.

Ms. Manju Sethi: Certainly, they do. I'll go back to the checklist and what was indicated on there that I covered in the talk. They have addressed two factors, the primers and the probes, and they have indicated that the publishing of those sequences is essential and desirable respectively. It is very, very highly encouraged that commercially available primers and probes have published sequences.

Sean Sanders: Uh-hum.

Dr. Gregory Shipley: So I've ranted for years against all of the major players in this game that they don't publish the PCR efficiency in the lower limit of detection. I mean, you're buying this assay from them. They should be able to tell you that. But then, more recently and perhaps more clearly, if you consider the conundrum that the company is under, so let's take Applied Biosciences, just, sorry, as an example.

Ms. Manju Sethi: No.

Dr. Gregory Shipley: So they make a lot of -- they don't make a lot of instruments but they also have a lot of competing instruments, a new one I understand coming

soon. And there's multiple -- there's SYBR and probe-based master mixes, all these variables, and then there's the person setting up the assay. So there are a lot of variables into determining those factors, okay? And so what I've encouraged them to do is say take one instrument, one master mix with your assay, and publish this information. But of course, in the case of Applied, they have a bazillion assays, and to go back and get all of these for everyone would be cost prohibitive.

So then, the onus goes on to the investigator then to get this information using their whatever master mix they're using, using the person they're using which is a variable that people don't appreciate, the instrument that they have, and then publish that data, and then that will be the data for that assay.

Sean Sanders: Uh-hum. So one of our viewers asks, is it appropriate for a researcher to simply say a method follows and meets all MIQE guidelines in their publications, or is there some kind of board overseeing this or some group of people that are looking at those?

Dr. Stephen Bustin: Well, there is the board but I think the reviewer should have access to primary data.

Sean Sanders: Okay.

Dr. Gregory Shipley: That would be my feeling.

Sean Sanders: And should ask for that?

Dr. Stephen Bustin: And it should be submitted with the paper.

Sean Sanders: Uh-huh.

Dr. Gregory Shipley: Yeah. It could be submitted to supplementals. I see that all the time when we review papers. Yeah.

Dr. Stephen Bustin: We have just published an editorial in BMC Molecular Biology, and this is a slight condensation of the MIQE guidelines and we have as an online submission process and includes a table that people can fill in with relevant information for their review.

Sean Sanders: To make it nice and easy.

Dr. Stephen Bustin: Yeah.

Dr. Gregory Shipley: Or maybe make an analogy and say, “Didn’t they tell the buyers of those junk bonds that they were all okay? We follow all the guidelines. It’s okay and…”

[Crosstalk]

Sean Sanders: Right, exactly. Trust us. Yeah.

Dr. Gregory Shipley: So science isn’t about -- the word “assume” should be erased from your vocabulary, number one. I mean, we all say it but you really can’t assume anything. You don’t know till you know is Greg’s rule number one in research. And secondly, you need to know that for yourself. You want to know that and so you should be able to show that. If you did it, you should be able to show it at the time of submission.

Sean Sanders: Uh-hum. So this maybe segues nicely into our next question which is what do you see are the greatest hurdles right now for broad acceptance of the MIQE guidelines or at least broader acceptance amongst researchers? Dr. Bustin?

Dr. Stephen Bustin: As scientists, we live by publication. So if we can get something published then that is the aim of a lot of people. That’s widening their work. It turns out that a lot of editors, journal editors, are shying away from accepting the responsibility of needing. What they’re saying is that there’s no groundswell for using MIQE so we will not suggest that people use these guidelines. I think that it’s going to be essential that journal editors realize that the papers that are being produced in their journals are possibly not producing data or not producing results, and reporting was also appropriate and this could affect the quality of their journal. So it’s in their own interest to make sure something simple as a set of guidelines that will ensure at least this technology or this technique is done appropriately and people follow these guidelines. And I think once journal editors get that lead then people will follow. I think it’s a kind of a chicken and egg situation.

Sean Sanders: Right.

Dr. Stephen Bustin: For BMC, I think the editors and the journals are beginning to understand and have taken the responsibility, have taken the first step. And I think really, journals such as Science, such as Nature and Cell really need to do it as well, and I think it’s scandalous that they don’t.

Sean Sanders: Uh-hum.

Dr. Gregory Shipley: Yes. And I would mention Clinical Chemistry is another one who has just recently adopted a form of the MIQE guidelines and they are also going to require them, as well as for microarray and some others.

[0:50:04]

So first of all, this is not the only technology. We're not setting ourselves up here as gods and saying, "You must do this." We're trying to present from our experience, and there's lots of us as authors on this paper, okay, from our experience the easiest and best way to perform this technique, and if you follow those guidelines, you will have a good result and a credible result, and isn't that what we're really all after in the meantime?

Sean Sanders: Right.

Dr. Stephen Bustin: NAR as well.

Dr. Gregory Shipley: Yes, NAR is also…

Dr. Stephen Bustin: Encouraging.

Dr. Gregory Shipley: Yeah.

Sean Sanders: So that three journals right now?

Dr. Stephen Bustin: Well, BMC I think is a multiple.

Sean Sanders: A multiple journal.

Dr. Stephen Bustin: Yeah.

Sean Sanders: Okay, good. That's great.

So actually a follow-up question on that is do you think there's any ways through the reviewers that some changes can be made for instance if reviewers on papers, even if the editors don't demand it, the reviewers are asking for these guidelines to be used? Do you think that's fair?

Dr. Gregory Shipley: Well, I mean I see papers from multiple journals. Presumably they want me to go in and look at the real-time part because it's an essential part of the dataset that's being presented. And so I go in and look at the technical underbelly as you will first, and mostly, that's where the comments come from. And I can tell you, I've passed these many papers through without being scathed a little bit. There are some excellent

papers out there. They just need one or two more things to round it out and I honestly just wait for them to do that. I'm not -- yeah.

Sean Sanders: Uh-hum, uh-hum.

Ms. Manju Sethi: Do the papers actually -- if I could ask a question. Do the papers actually indicate that they have been reviewed and are MIQE compliant if you will?

Dr. Gregory Shipley: They do mention that several times except they don't quite follow 100% through, and embarrassingly, some of those have authors on the papers that are in fact authors on the MIQE guidelines themselves. And so, you know, it's something -- once you get used to doing this correctly, it becomes second nature.

Sean Sanders: Right.

Dr. Gregory Shipley: We've been doing this in the core lab for 14 years now, this sort of thing, you know, but even we're not perfect, you know. We're also having to modify our methods a little bit so don't you think I'm sitting up here like we've always been doing this the best.

Sean Sanders: So this viewer says, "I've seen some companies advertising diagnostic kits that use qPCR. Are they adhering to these guidelines?" And I know Dr. Bustin did touch on this but maybe Ms. Sethi, you can address this. Is this something you've seen at other companies?

Ms. Manju Sethi: Well, I think we're on a journey right now and part of it is education. You had asked the question about hurdles, and in my opinion, there might be some lack of knowledge out there, people not aware of the MIQE guidelines. So I think the task for all of us in our own way, certainly a company like ThermoFisher Scientific, that's why we're doing this, is educating people who are working with qPCR, whether it be, you know, research and publication view or in molecular diagnostics that there are these guidelines, there are these tools available to them to conduct qPCR with transparency and confidence in results. So from our standpoint, it's education, making sure that people know and complying with it for our own instruments and assays.

Sean Sanders: Uh-hum. Now, what about data analysis? Does the MIQE guidelines touch on that at all?

Dr. Stephen Bustin: There's a lot of information on data analysis. As I said, data analysis is kind of a gatekeeper because inappropriate data analysis makes

everything else new redundant. And so there are a number of software programs available. I've tackled this. We use a couple. I mentioned qbasePLUS. The advantage these programs have is that they streamline the process of taking a raw result and filtering it through a series of user-definable parameters. So for example, you can define your error. You can define what would you accept, what variation between replicas do you accept, how many reference do you use, and so on. You can take these error calculations through the whole procedure and end up with a set of results that make sense, and you can trace and anyone can trace how you arrive at the result. So GenEx was known as another program that does that.

And so I think use of these programs helps and so MIQE encourages that. Bio-Rad for example, with each instrument they sell, they provide a copy of qbasePLUS. So that's a huge selling point for them I think because it means that people have access to a good instrument and the actual analysis of the data. So I think that I certainly encourage that.

Dr. Gregory Shipley: Applied has a free access program called DataAssist which I am soon going to learn how to use, but they have a more sophisticated program called StatMiner, which of course you have to purchase. And if you didn't buy a Bio-Rad instrument, you have to have a qbasePLUS, but it is an excellent program, and luckily, I have access to that as well, as well as GenEx from MultiD.

[0:55:12]

So there are some very good programs out there I think. I would encourage more companies to get into this idea that just because you have these raw Cq values, you know, what do I do with it now is what's everybody's question. And to really satisfy the guidelines, you need some help. The Excel spreadsheet, unless you're really savvy, it is not going to cut it.

Sean Sanders: Uh-hum.

Dr. Stephen Bustin: One last thing about GenEx is you can play around with the data and it gives you beautiful graphs and things. So it actually visualizes your data and you can see whether things are right or wrong. So people should try and use these programs.

Sean Sanders: Are there any other resources that you know of that you could point people to online? I know we were talking offline about other company websites that provide good information.

Dr. Stephen Bustin: Well, Bio has such a very nice primer design program as does Primer-BLAST is quite a good program as well.

Dr. Gregory Shipley: Yes, yes, and IDT now has one as well.

Dr. Stephen Bustin: And one I like to use for me is Plexor Design System because they use multiplexing so you can design mass multiplexes. You don't have to use the Plexor System but it gives you a nice set of primers that may well work together.

Sean Sanders: Okay.

Dr. Stephen Bustin: So that's nice, another nice website to use.

Dr. Gregory Shipley: Uh-huh, yeah, yeah.

Sean Sanders: Great. Well, we're coming to the end of our hour so I'm going to give you one last question, and that's just looking towards the future, what is your hope for the MIQE guidelines and maybe qPCR in general into the future? Where do you see things going and, you know, how hopeful are you that these guidelines are going to be accepted broadly? So, why don't we start with Dr. Bustin and we'll work our way down?

Dr. Stephen Bustin: Well, I think qPCR is going down the fast and faster, high and higher throughput using less and less material path. So that poses huge challenges for the future I think, huge excitement or huge challenges, and MIQE doesn't really address any of these issues. So I think we need to consider what's happening there. But I consider that in 5 years' time we will still be using real-time PCR because it is, compared to next-generation sequencing for example, far easier and far less bioinformatics intensive.

Sean Sanders: Uh-hum, sure. Dr. Shipley?

Dr. Gregory Shipley: Yeah. I think most of my customers are interested in knowing about a subset of genes because they've either run on microarray or perhaps they are coming from a next-gen experience, but now, they want to get under the hood. And, you know, validation is the name of the game, whether you're doing a screening with microRNAs, you're using some of these new array plates that are available, which is a whole another ball of wax we haven't even uncovered here in terms of issues as to how to use those. All these products are coming on the market, and as Stephen said, I mean, Roche has their 1536 out now. I'm sure that the volumes are just

going to get smaller and smaller and the throughput is going to get higher and higher. And just like with next-gen sequencing, you know, we're all going to run into this bioinformatic roadblock of "Oh, my goodness. It's great I have all these data, but now how do I deal with it?" So if you can deal with a 96-well plate and you're comfortable with that, then expanding won't be so bad. But oh, my goodness, this is going to be interesting.

Sean Sanders: Ms. Sethi?

Ms. Manju Sethi: From a company standpoint, we are keeping our ear to the ground. We know qPCR is a powerful tool and we're making sure that we try to stay ahead with leading technologies. Dr. Bustin mentioned small volumes. Things like that are very important to us so we're just trying to stay ahead of the game.

Sean Sanders: Great. Well, we're unfortunately almost out of time for this webinar so I'd like to thank our panelists for being with us today and for generously sharing their knowledge and expertise: Dr. Stephen Bustin from Barts and the London School of Medicine and Dentistry, Dr. Greg Shipley from the University of Texas Health Science Center, and Ms. Manju Sethi from Thermo Scientific. Also, just a quick thanks for being with us again.

And please hold on, all of our viewers. Stay tuned for just a couple of more minutes as we're going to have a very short presentation that will point you to some exciting resources that are related to today's webinar and might expand on some of the things you've already seen. So Ms. Sethi, the floor is yours.

Ms. Manju Sethi: Thank you, Sean.

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In keeping with today's educational theme if you will for qPCR and science in general, I just wanted to introduce you to the Journal of Visualized Experiments. It's called JoVE. And in my own paraphrasing, it's like a scientific YouTube. It's a wealth of information. You can see it. It's just how today's generation does it. They don't want to read it necessarily. They want to see it. It is peer-reviewed and it is a PubMed-indexed journal and in video format.

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And I just want to tell you about two that are relevant to some of the products you heard today. There is a video on quantitative real-time PCR using Thermo Scientific Solaris qPCR assays that we talked about. It gives you a lot more information about the product. You can learn more. We won't take your time here for that.

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There is also a microvolume protein concentration determination video using the NanoDrop spectrophotometer that you also heard about today. This particular video deals with quantification of protein using a couple of methods and there's a new one coming out for the quantification of nucleic acid. So we hope you enjoy JoVE in general and these two in particular. Thank you very much.

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Sean Sanders: Great. Thanks, Ms. Sethi, and thank you once again to our speakers. It's been really great having you here and we thank you for the knowledge you've shared with us.

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Also a big thank you to our viewers for the questions that you submitted. We did get a lot in today and apologies if we didn't have time to get to yours. Please go to the URL at the bottom of your slide viewer now to learn more about products related to today's discussion and look out for more webinars from *Science* available at www.sciencemag.org/webinar. This webinar will be made available to view again as an on-demand video within approximately 48 hours from now. Please share your thoughts with us about the webinar by sending an email to the address now up in your slide viewer; webinar@aaas.org.

Thanks to the panel and to Thermo Scientific NanoDrop and Solaris Products for their kind sponsorship of today's educational seminar.

Goodbye.

[1:01:18] **End of Audio**