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Assessment of Digital PCR as a Primary Reference Measurement Procedure to Support Advances in Precision Medicine

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Guest: Dr. Jim Huggett is from the School of Biosciences and Medicine, Faculty of Health and Medical Science at the University of Surrey in the United Kingdom.

Bob Barrett: This is a podcast from *Clinical Chemistry,* sponsored by the Department of Laboratory Medicine at Boston Children's Hospital. I am Bob Barrett.

With the advent of precision or personalized medicine, treatment decisions for patients with cancer are increasingly being made based on their germline and tumor sequence variation. Tumor genotyping is required to reveal nucleotide sequence variants that will confer benefit from certain therapies. But how accurate is this testing?

In clinical chemistry, benchmarking of test and laboratory performance is often provided by using a series of procedures beginning with the primary reference measurement procedure that is the most accurate approach and is used to assign values to primary calibrators. In molecular testing, only a limited number of reference materials are available, and because no reference measurement procedures exist, the traceability of patient results is limited. However, digital PCR is a technique that counts individual DNA molecules without the need for a calibrator and is capable of high sensitivity in the detection of low-frequency variants, and may hold the key.

A recent study to address that possibility appeared in the September 2018 issue of *Clinical Chemistry*. It was an assessment of digital PCR as a primary reference measurement procedure to support advances in precision medicine.

We're pleased to have one of the authors of that paper as our guest in this podcast. Dr. Jim Huggett is at the School of Biosciences and Medicine, Faculty of Health and Medical Science, University of Surrey Guildford in the United Kingdom. So, first of all, Dr. Huggett, what is a primary reference measurement procedure, and what makes it so special?

Dr. Jim Huggett: Okay, so, probably the best way to begin that is to consider what a reference measurement procedure is on its own.

Reference measurement procedures support accurate measurement, and they're used to provide values on reference materials or, in cases on clinical samples, provide reference ranges. They serve to support reproducible measurement as they're traceable. This traceability can be to reference materials, they can be actually to the individual method and consensus units can be used, or at their most accurate, they are traceable to the Système international d'unités, which is the SI unit. Primary reference measurement procedures enable the latter.

Now, to quote the Bureau of Weights and Measures, the BIPM, which is based in Paris, where they have the kilogram, they are methods that have the highest metrological quality, whose operation can be completely described and understood for which a complete uncertainty statement can be described in terms of SI units. Importantly, the results are therefore accepted without the need to reference to another standard.

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Bob Barrett: And why would digital PCR be suitable as a primary reference measurement procedure?

Dr. Jim Huggett: Okay. So, if we think of PCR and particularly what we call classic quantitative PCR, quantitative PCR is able to quantify DNA molecules in real time by measuring the increasing fluorescence as the marker of the amplification procedure as the reaction proceeds.

The initial concentration is influenced by how much DNA is present. So, initial concentration of the DNA will influence when the fluorescence increases, such that if a sample has more DNA, it will generate a signal much earlier in the reaction. Now, while this relationship holds, through accurate measurement, QPCR requires a calibration to something, and this is either to a reference standard or another sample.

Digital PCR uses the same concept as QPCR from a reagent point of view, but it differs in a critical way. In digital PCR, the DNA molecules are partitioned into individual smaller partitions or little reactions. And each one of those contains an individual or small number of DNA molecules. And when we quantify, we simply count the number of positive and negative reactions. Now, as long as A) you can be confident that if a DNA molecule is present it would generate a positive signal, and B) you have an accurate idea of the volume of those partitions in which the experiment is performing, you potentially have a technique that can be accurately counting the DNA molecules and this would be SI traceable. And in this case, it would be counting the molecules and traceable to the unit 1.

Bob Barrett: Okay, let's talk about in vitro diagnostic tests. Why do they need primary reference measurement procedures?

Dr. Jim Huggett: Well, as the use of molecular in vitro diagnostic tests is growing, and it is growing quite increasingly especially in the United States, there is an increasing need for the infrastructure to support their continued development and robust application of their use. This is things like accreditation of the tests, proficiency of their use. In other areas, this is supported by external quality assessment schemes. And where the targets are simple and they're not many, then reference materials can be used to support this with the material being the source of the traceability. However, as the targets become more diverse, such as in precision medicine where multiple genetic variants may need to be measured and different ones in different laboratories, traceability to a material becomes much more challenging, because you need many, many more materials.

And so, in this scenario, a reference method that can provide the measurement of multiple targets offers an alternative source of traceability. As the community demands ever more sensitive detection and an increasingly improved quantification, the role of an SI traceable method becomes valuable, because you get a much better idea of the performance of the diagnostic test in terms of things like its limited detection and perhaps limited quantification. It also provides you with—potentially gives you a better understanding—of the actual molecules' behavior in the patient from an accuracy point of view. So, the number of single nucleotide variants that may occur in a patient sample is more accurately determined, so it gives you a better idea of the dynamics of what you're measuring.

Bob Barrett: There must be challenges in developing reference measurement procedures, particularly in nucleic acid sequencing. Can you talk about those?

Dr. Jim Huggett: Certainly. Now, in the case of the digital PCR, these two assumptions I'd spoke about early on, that of the volume of the individual partitions and whether a DNA molecule that is present is being amplified, are the two key things to demonstrate. Now, the volume of the partitions is comparatively easy to be done and with some high accuracy physical optical measurements, has been demonstrated by a number of measurement institutes for a number of instruments.

Whether a DNA molecule is being amplified is more challenging. And so, you have the issue, let's assume there are a hundred molecules, are we amplifying all 100 molecules, or an estimate of all 100 molecules in appropriate uncertainty? And this is a bit of a circular

problem, because this digital PCR is the first method of its kind to be able to measure so accurately what you compare it to. The methods must also be reproducible across different laboratories and ideally with different instruments. So, we and others in earlier publications of research demonstrated the high reproducibility of digital PCR even between instruments, which is really important.

Importantly as well, is that this reproducibility is without calibration. And this is an absolute quantification of the DNA molecule independent of calibration. And it is completely unique to digital PCR. It simply cannot be achieved with other molecular methods. The fact that they're all reproducible and the methods are agreeing does not however prove that the method is correct in providing an accurate, SI-traceable result. To do this, we need to compare with another SI-traceable orthogonal method. And this is essentially what this work has demonstrated when measuring single nucleotide or a single-nucleotide variant. And this gives us much greater confidence that the method is measuring the hundred molecules with a greater accuracy.

Bob Barrett: Finally, Dr. Huggett, what routes do we need to follow to ensure this work will impact the development and application of in vitro diagnostic tests?

Dr. Jim Huggett: So, this is really early days, this is the early stages. At this stage, we are continuing to investigate the wider application of digital PCR as a primary reference measurement procedure. And this has included the submission of the protocol associated with the paper as the first ever reference measurement procedure for nucleic acid measurement to the Joint Committee for Traceability in Laboratory Medicine Database. This provides tools to support the IVD industry to meet traceability requirements. But the work continues, and it is a truly collaborative effort at an international level.

The Nucleic Acid Working Group at the Consultative Committee for the Amount of Substance, the CCQM, which is of the BIPM, is championing this research by supporting pilot studies to investigate high accuracy measurement of other cancer mutations, as well as viral nucleic acid targets, in which digital PCR can be applied alongside a range of orthogonal methods to really understand and investigate its sources of uncertainty and describe its accuracy.

Wider impact will also require the investigation and description of other sources of uncertainty. Importantly, the work that we're describing here is only measuring the DNA. Indirect sources of uncertainty are still there and often predominate, such as uncertainty associated with the

extraction procedure, which is necessary to purify and concentrate the DNA for it to be analyzed using molecular methods. However, with an SI-traceable method, we're in a very strong position to really evaluate these pre-analytical steps with a much higher level of accuracy. And so, we are in a much greater position to support the most accurate amplification and translation of molecular IVD tests to have the maximum impact on patient care.

Bob Barrett: And that was Dr. Jim Huggett from the School of Biosciences and Medicine, Faculty of Health and Medical Science at the University of Surrey in the United Kingdom. He has been our guest in this podcast on digital PCR as a primary reference measurement procedure to support advances in precision medicine. That article appears in the September 2018 issue of *Clinical Chemistry*. I'm Bob Barrett. Thanks for listening!