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Digital PCR as a Novel Technology and Its Potential Implications for Molecular Diagnostics

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Guest:
Dr. Jim Huggett is Science Leader, Nucleic Acid Metrology at LGC, an international life sciences company based in the United Kingdom.

Bob Barrett: This is the podcast from *Clinical Chemistry*. I am Bob Barrett.

The latest incarnation of polymerase chain reaction, Digital PCR, takes three decades of development in enzyme chemistry and assay design and applies them with formidable precision and sensitivity.

Digital PCR was achieved by performing a limiting delusion of DNA into a succession of individual PCR reactions.

In the December issue of *Clinical Chemistry,* two studies have demonstrated clinical applications of Digital PCR technology for measuring circulating (cell-free) nucleic acids.

These papers were accompanied by an editorial on the topic by Jim Huggett and Alexandra Whale, of the Molecular and Cell Biology Department at LGC, an international life sciences measurement and testing company based in the United Kingdom.

Dr. Huggett is our guest in this podcast. Doctor, simply, just what is Digital PCR?

Dr. Jim Huggett: Okay so, Digital PCR is a method that takes advantage of the chemistries associated with PCR and Real-Time PCR and essentially allows one to count DNA molecules.

And it does this by allowing you to perform a series of limiting dilutions, where you will take the template of DNA of interest and you will dilute that across a large number of PCR reactions, so that, due to the random distribution, some of those PCR reactions are actually negative, they do not contain any DNA molecules.

And this concept is quite common in a number of biological study areas, like cell biology where people will look for individual clones; and the distribution of the DNA when you perform this limiting dilution follows a particular pattern and

you can apply statistics to predict the number of molecules that are present in the individual reaction.

And that differs with Real-Time PCR whereby you basically perform a PCR reaction and watch the amplification in real time, in that Real-Time PCR requires some kind of calibration curve to quantify the abundance of the template that you are actually measuring.

Digital PCR was originally termed by the Bert Vogelstein and Kenneth Kinzler in a publication in 1999. However, the actual process of limiting dilution using the PCR methods, occurred much, much earlier and the original publication that has been discussed, was actually published in 1988 by Randall Saiki and that also in that authorship contained both Kary Mullis and Russell Higuchi.

So there was quite a prestigious authorship on that particular paper. And Digital PCR was being used in the early 90s to perform a number of methods of quantification. And it was used by the late Alec Morley to look into the abundance of PCR able and a number of other people were using it as a quantitative tool.

The problem with Digital PCR in the early 90s was that it was a cumbersome method. You have to dilute the template across a number of different normal PCR reactions, so for an individual sample you may dilute across 96 different reactions. So there was quite a lot of work for an individual template.

And so when Real-Time PCR came along, it basically, published in 1992, it basically took over and was the new method, a much, much simpler method for quantification of the template DNA.

And it is only now that with the development of methods, technologies to approach nanofluidics and similar time emulsion chemistries to allow us to actually perform multiple reactions in a much more automated fashion that Digital PCR is really taking off again.

Bob Barrett: Well, doctor, tell us the benefits of Digital PCR when compared with conventional and real time quantitative PCR?

Dr. Jim Huggett: Well, there are three key benefits that are frequently referenced and there is a fourth which I would argue is possibly the main advantage that will perhaps stamp Digital PCR on and to be applied clinically.

The first one will be that you have a much more predictable and precise quantification of a particular template than you

do with Real-Time PCR. And to add to this, it does not require a standard curve to provide a quantitative value.

The random distribution is very, very predictable and it provides you with a given variance and this appears in the work we have done to follow the mathematical rule.

So it is very, very, very precise and this prediction, it is much more easier to design experiments to apply power calculations because the Digital PCR, unlike Real-Time, tends to follow this distribution, whereas Real-Time PCR, the precision of Real-Time PCR is very much dependant upon the optimization of the reaction.

Now it's fair to say in this current day and age that Real-Time PCR is very, very precise as well, but we find it is not quite as precise as Digital PCR.

Now this improved precision leads to the second major advantage, and that is its ability to measure very, very subtle differences and so you are trying to measure one sample to another and you can measure much smaller differences than you can with Real-Time PCR.

And where this can be very, very useful is if you are looking for example at copy number variations in say cancer genome instabilities or maybe looking at fetal aneuploidy, and where you are looking at that perhaps taking a blood sample from, in the case of the fetal example, from the mother.

And so you can measure potentially, and the research has been performed to see where this will be possible, you have the potential to measure an imbalance that, for example, for Trisomy 21 that causes Down Syndrome, there is a potential, you can actually do a blood test and just determine whether the fetus actually has Trisomy 21.

The third ability and the third big benefit of Digital PCR is its ability to, because of the limiting dilution, to actually dilute out rare mutants, facilitate their detection.

And so here you may have a scenario, a good example is when you are screening, say, again using blood, to try and predict the mutation or status of a solid tumor for example in colon cancer.

And so we are looking for this mutation that may guide therapy from a blood test you have to find this mutation when there is predominantly normal mutant sequences – normal wild-type sequences, sorry, and so when using methods like Real-Time PCR, this could be challenging because the methods that actually pick up the mutant

sequence will also pick up the wild-type sequence at a much, much lower frequency.

Now, that lower frequency becomes a problem when the wild-type sequence is predominant, and so consequently digital, by performing this dilution, reduces the background of the wild-type sequence and increases the chance of you picking up a mutant sequence.

And that can also be used potentially, and has been used, to pick up fetal mutations from maternal blood, and it can also be used, for example, in tracking resistance in say particular for example pathogen resistance in a particular infection.

For example you could perhaps use this to look at development permutations of things like HIV or influenza, and so you have the potential, have a much more sensitive method than is currently available in the Real-Time PCR.

Now, those are the three advantages that are frequently reported, but the fourth big advantage and certainly where I work, we are very interested in the standardization of different methodologies, is that we believe Digital PCR will be much simpler to perform and get reproducibility.

And by that I mean if I take a piece of DNA and I analyze it in London, and then I hand it to you, Bob, and you analyze it in the US, well, it's going to be much easier for us to get a very similar result.

With Real-Time PCR this is much more challenging because there are certainly more variables and so consequently we have to employ quite challenging calibrators which do the job, they work very, very well, and so different laboratories are able to get similar results, but it is a challenge, there is no two ways about it.

Whereas with Digital PCR we believe it will be much, much simpler for different laboratories to get similar results and this will be a major, major advantage when performing in the clinical diagnostic measurements and quantifying.

And there are some additional advantages with digital like looking at the relationship in different sequences so you may have advantages when performing different haplotypes or if you are interested in the integration of viruses into host genomes.

Bob Barrett: Those are the advantages. There have to be disadvantages, what are those?

Dr. Jim Huggett: Certainly, now the obvious early disadvantages are what I call any disadvantage that a new technology has, certainly

when it's competing with established methods, and so Digital PCR is certainly currently a lot more expensive than Real-Time PCR. And at the same time, it takes longer to perform the reaction and depending on the which instrument you have, that can be a little bit longer or considerably longer.

The current setups do not have the same ease of dynamic range compared to Real-Time PCR. The Real-Time PCR reaction, it is very simple with the Real-Time PCR reaction to get over nine orders of magnitude. And that nine orders of magnitude you get a very, very similar variance on that measurement which is actually quite incredible in clinical measurement. Real-Time PCR really is fantastic for that.

Digital PCR is limited by the number of reactions you perform and so to get nine logs with Digital PCR you can imagine you will need a huge number of reactions. You don't quite need nine logs of reactions but you do need a considerable number and so this obviously has challenges associated with whichever format you are looking at, so a big, big challenge.

There is also an underlying issue and that is the reaction volume you have at the moment. Because you are limited by either the chip or the emulsion chemistry setup you have, you do not have the same scalability that you do with the Real-Time PCR reaction.

And so for example, where Digital PCR may be more sensitive than Real-Time PCR when comparing very, very small amounts of DNA, so there is a possibility if the volumes are matched, the Digital PCR, for example measuring a virus like human cytomegalovirus, could actually be more sensitive.

The problem we have at the moment with Digital PCR is that my clinical collaborators that you see at University College London Hospital, they will run 50-100 microliters of Real-Time PCR reactions, and in that they will put between 20 and 30 microliters of the clinical extract.

Well at the moment Digital PCR reactions are the most, one of the highest volume I believe to be about 50 microliters, but certainly the ones we use have less than 20 microliters and one of the instruments we use is very popular, has actually 600 nanoliter reaction volume.

So even though that, volume for volume, may actually be more sensitive, you can't physically get the same amount of clinical extract into the reaction, that has a big limit on its actually sensitivity. And so until we actually have instruments to facilitate much larger volumes, we will not be

able to compete with the sensitivity of Real-Time PCR which let's face it, has set the benchmark.

The other big challenge with digital which probably will not go away, and it may be rendered more irrelevant but it will not go away when directly compared with Real-Time, is the complexity of setting your reaction up.

Now whether you are using a chip-based method and chip-based methods use foliage chips to perform the dilution so you will have a prefabricated chip with a number of very small reaction volumes or whether you are using emulsion chemistry and these are essentially cells which isolate your individual reaction and you can form many, many thousands of them and these have been developed in both with next generation sequencing technology in laboratory preparation.

Whichever method, you are going to have a challenge because you have got to generate this number of reactions and as I said you need 100 million to be competing with the Real-Time PCR. This is going to be a complexity that will not go away. It may be simplified and hopefully it will but it will remain a challenge when compared to Real-Time PCR.

Bob Barrett: Why are minimum information for publication of quantitative Real-Time PCR experiment guidelines needed for Digital PCR?

Dr. Jim Huggett: Okay, so the original guidelines, which have the acronym MIQE which I'll use from now on as it's simpler, were published because we felt there was an awful lot of work that was published without providing comprehensive information on what was performed and how the Real-Time PCR reaction was performed.

And this was originally presumably due because the lack of information was available when one published, or lack of space rather, when one published and so it was not easy to get all the information present.

This is much less likely now because we have a lot of supplementary information published online, and so it really is very easy for people to publish all the information.

This is also combined with the fact that there is a lot of kits available to perform these molecular methods, and so consequently often you did not know exactly what it was you were using, and so this limited a lot of the information that was available.

And so what MIQE has done is championed people providing as much information as possible and to be fair has really got the companies to agree to provide much, much more

information and I think it's fair to say that the companies are some of the bigger uptakes for the MIQE guidelines and we are very, very pleased that that's occurred.

Now why does digital need this? Well, it's generally agreed amongst the fraternity of Real-Time PCR that the MIQE guidelines really came too late, because a lot of this information, and a lot of people were publishing without providing this information.

So the group of us decided that we would like to get in there early with Digital PCR. Now Digital PCR is arguably more reproducible than Real-Time PCR and I believe that will certainly be the case, however there are some things that need to be included in the publication that maybe overlooked that will make it much easier for me to assess your work for example.

And so we put these together both to explain the nuance of the Digital PCR to the people who – to the uninitiated so to speak, but also to provide a list of detail that we felt really should be included in the actual publication, so that when people publish they make sure that that is comprehensively detailed and that then allows both reviewers and editors to also assess the paper and facilitate reproducibility, essentially.

Bob Barrett: Well, finally doctor, will next generation sequencing simply eclipse any potential role that Digital PCR might be able to offer?

Dr. Jim Huggett: Now this is a very good question and one that gets a lot of debates, at a lot of the meetings and depending on whether it's a sequencing meeting and Digital PCR meeting, I guess you have a different answer.

Personally I think one of the real advantages of Digital PCR is it simply takes advantage of the 30 years' development that we had with the polymerase chain reaction and applies it in a different format.

When Real-Time PCR came along some 15 years ago or so, it really demanded that the enzyme manufacturers get their act together and produce really, really good enzymes, and there was a real race, and to be fair they have really done this, with Real-Time PCR, to ensure that these enzymes were working very well, were highly reproducible, gave you very small variances.

Well, Digital PCR simply takes that technology and performs the limiting dilution, but you don't really have to do anything with the enzyme chemistry apart and it's really just the setup of a technology getting that complexity of the

limiting dilution possible that is the challenge and to be fair that there are several companies that are actually doing that now, they have done it and it works. So I really believe digital has the edge because it's simpler.

Now with regards to next generation sequencing, I really do believe that that will be the future and certainly single molecule sequencing will replace Digital PCR, PCR at some point, because we need to remember that the polymerase chain reaction is really a finesse and it's a finesse of the fact that our optical methods are really poor.

We cannot really measure DNA unless we have tens of millions of copies plus and so PCR allows us to sidestep that problem by going in and choosing the molecule we are interested in and generating billions of copies of it.

Well as soon as the next generation sequencing, single molecule sequencing, bearing in mind current next generation sequencing still requires that amplification step to generate enough material because even the instruments that are out there they still cannot measure individual molecules without some kind of amplification to get around this problem of our optics.

So when that eventually is cracked, yes I do believe that the polymerase chain reaction will certainly perhaps lose its role in quantification of detection.

However, when will that be? That's the big question. And I know there are people who profess that they reckon that the next generation sequencing will take over in next five years. I will be surprised, pleasantly surprised, but I will be very surprised if that is the case.

I certainly think that the likes of Digital PCR still has an awful lot to offer and certainly may well actually support, and actually to be fair are already supporting, the application of next generation sequencing technology.

So I think it's going to be an interesting next decade and perhaps we can have a chat then and see what happens.

Bob Barrett: Dr. Jim Huggett is Science Leader, Nucleic Acid Metrology at LGC, an international life sciences company based in the United Kingdom. He has been our guest in this podcast from *Clinical Chemistry* on Digital PCR.
I'm Bob Barrett. Thanks for listening.