Steve Lewis 00:10
Welcome to Speaking of Mol Bio, a podcast series from Thermo Fisher scientific about molecular biology and its trending applications in life sciences. I'm Steve Lewis, and today I'm excited to bring to you the first Mol Bio Minutes mini episode of season four. Today, we welcome our own Dr. Andrea Hunger. Andrea has a wealth of experience across endpoint PCR, qPCR and digital PCR, using these tools in both academic and industry settings. In this episode, she walks us through the strengths and ideal use cases for each PCR method, offering a clear and practical roadmap for both new and experienced users. We hope you enjoy.

Andrea Hunger, PhD 00:57
Hello. My name is Andrea Hunger, and currently I am a scientist three on the molecular biology specialty team at PPD. PPD is now a part of Thermo Fisher Scientific which many of you might have heard of. We at PPD are a CRO, or a contract research organization, and my team specializes in both qPCR and digital PCR for clients that are involved in cell and gene therapy. I also have a PhD in biochemistry from the University of Wisconsin - Madison, where my research focused on finding novel genetic regulators of the metabolic changes that occurred in both diabetes and in cancer. And as you might imagine from this background, I've used a lot of different kinds of PCR, and so I wanted to take a few minutes today to share with you, sort of a summary of what I've learned of these different types of PCR and what applications they're best suited for.

So to start out with just a really general introduction, PCR, or polymerase chain reaction, is both a specific and a sensitive tool for gene expression analysis. In my academic work, for example, we were looking at gene expression in a variety of different mouse tissues and human cell lines and many different contexts from many different applications. So that work exposed me to many different types of PCR, and that's the expertise that I'm hoping to share with you today. So starting with maybe the most simple type of PCR is an endpoint PCR, and this is really useful, and what I have used it for myself is for cloning and genotyping. So things where we really just need to know, is it present or is it not? This type of PCR doesn't give you a really granular quantitative answer. It's more like a yes or a no. So it's best for answering simple present, not present questions. But the advantages are that it's very quick and very simple to perform. It doesn't take a whole lot of training. You could get an undergrad to do it in an afternoon, very easily. Some limitations again, are that it's not very quantitative.

So if you need an answer that's quantitative, we'll have to use a different method. For example, you could use something like real time PCR or qPCR, where the Q stands for quantitative. This is a really useful method for when you actually need to know how much is in your sample. So there are two main different types of qPCR. You can use either a fluorescent dye or a probe. But in both cases, we're going to be generating amplification curves, and that's what enables the quantitation of your DNA and your starting material. For the dye based the dye is commonly a SYBR Green, which is what I've used in my academic lab. This is a really cost-effective way to do qPCR. It's a little bit less specific, so it can be sensitive to some off-target or non-template amplification. So in this case, your controls are going to be really important to helping interpret that data. We also can't multiplex when we're using a dye based, so that can be a drawback in some cases, but again, it's a really effective tool that I've used many times. The other option is probe based, which can be a higher upfront cost, but it can also be more specific and more accurate than some of the dye-based options. And it can be multiplexed. So it can be

a little bit higher of an upfront cost, but that can be useful in an application where you need to measure multiple targets in the same sample.

One thing to remember about qPCR, if you would like to make a quantitative comparison, is that you have to use a standard curve. So this is where you serially dilute a known amount of target DNA to create something like a calibration curve, and then you can measure the amplification of each dilution of that calibration curve along with your sample and use that data to determine what the concentration was in your sample or your unknown. That calibration curve is made of Ct, or cycle threshold values. This value is the PCR amplification cycle at which the fluorescent signal, that's created by the amplification of your DNA of interest, crosses that set threshold. And that threshold means that it's first reliably detected by the instrument. So the first time, we can say “Yes, that DNA is in your sample.” And so this gets to be a little bit counterintuitive, but a lower Ct value is a higher concentration. So it means that the DNA was amplified to that detectable threshold more quickly and there's more starting material. Because we're making these exponential measurements, because the amplification is exponential, this also gives us a really large dynamic range. So that's another advantage of qPCR is we can measure a big range of concentrations in the same assay. It can also be very high throughput, right. We can load up a 96-well or a 384-well plate and get through quite a few samples in one run.

The other thing I've noticed about qPCR in my own work is that many of the scientists around me, both in my academic lab and my lab now in industry, many of them have experience. qPCR has been out for a while. A lot of people around you might be really, really good resources to learn more about it. And there's also really good resources online, including validated databases of primers if you're working with mouse or human samples, so you don't necessarily have to worry about making your own primers. You can use some from other researchers that have been validated, tested, we know they work. So that can be a really good advantage, especially for someone starting out in qPCR, who maybe doesn't know exactly what parameters to optimize in their primer design. qPCR can also be a really fast and affordable method to get through a lot of different sample types. So for example, in my thesis research, we were quantifying the expression of something like 10 genes related to differentiation in 10 different tissues. And so qPCR was a really effective way for us to look at all of these different genes that are expressed at very different levels in different tissues. So it's a really useful technique for many researchers.

It does have a few disadvantages. We can make relative comparisons pretty easily, but again, if you want to make a quantitative measurement, you're going to need that standard curve, so we need to have a positive control that we can make a serial dilution of. One of the other disadvantages of qPCR is that, because we're making our measurement during that exponential amplification, the reactions can be sensitive to PCR inhibition, so anything that slows down that amplification can affect your measurement.

The final form of PCR that I'd like to tell you about today is digital PCR, and this is really useful for when you need to know exactly how much of something is present in your sample. For when you need an absolute quantification. And this is a really interesting, exciting, newer technology. It hasn't been around quite as long, but there are still a lot of resources to help you get started if it's something you find you're interested in. So this technology uses spatial separation done by microfluidic technology to separate

your reaction, your PCR reaction, so you're diluting your samples into small enough volumes that it is statistically likely that each small volume contains either one or zero copies of your DNA of interest. After that separation is complete, we then run the PCR to completion. So it's similar to endpoint PCR in this way. We're going to run the whole reaction and then do our detection after. So we're running to endpoint and then taking our measurement. And then at the end, your data is generated by counting the number of fluorescent, meaning positive, or non-fluorescent, meaning negative, micro chambers, and this is what allows us to provide the absolute concentration. And then Poisson statistics are used to establish a confidence interval for that data. And some of the advantages of a digital PCR is that we don't need to have a standard curve, because it uses direct quantification. So this is also really useful if you're working with a sample that has some things that might lead to PCR inhibition, because we're going to run that reaction to the endpoint and then take our measurement, so it's less sensitive to those inhibition effects than a qPCR would be. Another advantage is that it has really accurate quantification of very low abundance sequences. Something like needle in a haystack quantification. So where that signal might get lost in a qPCR. A digital PCR is really good at finding rare variants are really low, copy number material.

Some disadvantages is that because we're separating the amplification from the data generation phase, it can be slower because we're doing this as two discrete steps rather than at the same time. But when we say slower, you could still easily get it done in an afternoon. It can also be more expensive because of the reagents and the technology involved, and it can be more technically challenging to execute. But again, I learned how to do it in, I think two days when I started my new job. So there's still a pretty low barrier to entry, many resources available, and many experts in the field that you could turn to.

An example of a use case where a digital PCR would be really helpful, and where you kind of need that level of information would be something like identifying and quantifying rare mutations in liquid biopsies for cancer or genetic disease research. Sometimes we really need that level of information.

So to end with just a quick summary of the use cases for these different kinds of PCR, an endpoint PCR can be really useful if you just need a present not present. Answer like, “Did I clone the right gene? Did my PCR work at all?” Really useful for that. I like to think of it as a quick and dirty measurement. We just need a yes or a no and that will get us to the next step. qPCR is really a good option for high throughput applications or samples with a really large dynamic range, or when you only need to make a relative comparison, “Is there more in sample, A versus B?” And that's a good enough answer. Digital PCR then is best when you're quantifying a really low abundance target, or when you need an absolute quantification, you need to know exactly how many copies per microliter are in your sample. But when we talk about it this way, it can seem like one of these methods of PCR is good and one is bad or one is better. But really they can and should be used to complement each other. Maybe you start with an endpoint PCR to figure out if your gene of interest is there at all, and then you move to a qPCR, or a digital PCR to figure out how much is in your sample. But it would be a waste of time to start there if you didn't have a gene of interest in your sample at all. So it's important to consider that each type of PCR has different strengths, and they provide different information. So what really helped me in my PhD was to think about the final application. “What am I using this data for, right? What is my next experiment, and how much information do I need to get there?”

And I was hoping to end with just a little anecdote from the very end of my PhD. If you don't work in an academic research lab that is also a publicly accessible building, you might not know this, but we get a lot of sales representatives from different biotech companies walking through our lab spaces. They leave flyers and coupons. And I think a lot of us, especially when you get to your fourth, fifth, sixth year of your PhD, we don't listen as well as we did at the beginning, until they get to the part about the free lunch. I remember seeing a flyer as I was putting together my thesis that said, “Pizza lunch. Come learn about digital PCR.” And I just remember thinking that, “I had done so much qPCR. Why do I need to learn this new method, I just need to put my paper together and graduate.” And then fast forward to about a month later, and my job requires me to be an expert in digital PCR, so really, I should have gone to the seminar. And I think that's an important lesson to, even if you are an expert in your field, the field is always moving. So it's important to stay open to new ideas and new technology. And you never know when something that you just completely ignored because you were busy that day becomes something that you do every single day in your next role. If you made it all the way to the end, I would like to thank you so much for sharing your time with me, and I hope that you learned something new today.

Steve Lewis 12:18
That was Dr. Andrea Hunger, scientist three at Thermo Fisher Scientific. As always, for these Mol Bio Minutes mini episodes, we recommend that you check out the Episode Notes for links to the helpful resources that Andrea covered. We'll have another Mol Bio Minutes mini episode next month, but up before that will be a full interview episode with me. Stay tuned for that to drop, and until then, cheers and good science. Speaking of Mol Bio is produced by Matt Ferris, Sarah Briganti, and Matthew Stock.