UNITED STATES DISTRICT COURT
EASTERN DISTRICT OF NEW YORK

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STEPHEN BUSTIN AND DAVID CROWE
DISCUSS THE RT PCR
AS BROADCAST ON THE INFECTIOUS MYTH

APRIL 14, 2020

TRANSCRIBED BY CHIRON RETURN

FOR PLANET WAVES FM

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MR. CROWE:

We're addressing a very technical subject this week, RT-PCR. I think if you sit and listen in a quiet place and perhaps play one or two parts over again, you can learn a tremendous amount about this technology, which is currently being used as pretty much the only test for Covid 19 infection.

Professor Stephen Bustin is a world-renowned expert on quantitative PCR, and his research focuses on translating molecular techniques into practical, robust, and reliable tools for clinical and diagnostic use. He received a Ph.D. in molecular genetics from Trinity College in Dublin, working on fungal and bacterial pathogens.

Apart from numerous scientific papers, review articles, and book chapters aimed at improving the reproducibility and robustness of real-time, quantitative PCR, Professor Bustin has authored the books A to Zed or A to Z of Quantitative PCR in 2004, The PCR Revolution in 2011, and PCR Technology in 2013. He's been an expert witness in the U.K. High Court and also in a court in Washington D.C. He helped develop the MIQE guidelines that we will talk about today for use in reporting of qPCR and digital PCR.

Welcome to the show, Stephen.

MR. BUSTIN: Thank you very much.

MR. CROWE: And thank you for taking some time out of what in England is a holiday.

MR. BUSTIN: That's right, a bank holiday.

MR. CROWE: Yes. In Canada, we can't decide. I think it's a federal holiday but not a provincial holiday. So if you work for the government, the federal government, you get it off, and if you work for the provincial government, you don't.

MR. BUSTIN: Right.

MR. CROWE: So I think everybody knows that RT PCR -- and we'll get into more of the definition of this -- is really important in the current Corona Virus situation because it's being used as the testing methodology. And I don't think too many people understand the first thing about it, except those skilled in the art, as they say. So I've divided the discussion of the technology into four parts, so let me know if this makes sense.

One: Extraction of the RNA; two, conversion to complimentary DNA; three, PCR replication of DNA; and possibly four, sequencing.

MR. BUSTIN: Okay. Sequencing is probably not part of the RT PCR. But as I said, RT PCR is used for sequencing but sequencing is a separate technique.

MR. CROWE: Right. I do have some questions about that. It's not like a core part. You can do those three parts. They're sort of -- you have to do those first three parts and sequencing is kind of an optional thing.

MR. BUSTIN: Yes. RT QPCR itself is a technique in itself. The RNA sequencing is a separate technique which uses RT PCR.

MR. CROWE: Okay. And I'll ask you some questions about that, I hope, later. So one of the things I always found confusing, and you are very consistent in your use of terminology. You talk about QPCR, quantitative PCR, which is real-time, quantitative PCR, and then there's RT QPCR, which is reverse transcriptase PCR. Now, the problem is, there are two RT's and the reverse transcriptase I understand because that converts RNA to DNA, but what the does the RT real-time PCR mean?

MR. BUSTIN: Well, the MIQE guidelines define this. RT PCR simply means reverse transcription PCR. Real-time means that as opposed to an end-point assay, where you would run a gel and then look at the fluorescence that comes from the

get that you're looking at. In real time, you are monitoring the reaction as the PCR reaction progresses in real time. So you see an amplification plot that is -- that increases as you have more and more product being generated. And because it is in real time, it's called real-time PCR.

MR. CROWE: Okay, so however you measure the amount of DNA -- it seems a lot of people are using fluorescence.

MR. BUSTIN: Yes.

MR. CROWE: Then at each cycle, you would say, okay, now we have this much fluorescence, now we have this much.

MR. BUSTIN: That's right.

MR. CROWE: And then you could graph the change over time.

MR. BUSTIN: That's right.

MR. CROWE: Okay, that's pretty clear. Now, two other words that I think can be confusing, and I hope I'm going to get these right, are probe and primer. My understanding is a probe helps to detect the target RNA in the original sample, and the primer delimits the portion of the DNA that's to be replicated in the PCR step. Is that correct?

MR. BUSTIN: That is correct, but you can get a PCR reaction without a probe. So the primers themselves are

sufficient to generate your PCR product, which you can then detect with a non-specific dye.

MR. CROWE: Okay.

MR. BUSTIN: The probe simply adds the additional specificity that makes you more confident that whatever result you get is in fact the real result because you're detecting not a non-specific dye binding to something that might have given you an erroneous replication, but it has to be the actual application of product that you are interested in. So a probe is simply an additional insurance policy. It makes it more specific.

MR. CROWE: Yes, and this occurs during the RNA extraction phase?

MR. BUSTIN: No.

MR. CROWE: No?

MR. BUSTIN: No.

MR. CROWE: Okay.

MR. BUSTIN: The probe is entirely during the PCR step itself.

MR. CROWE: As well as the primers?

MR. BUSTIN: The primers are also at the PCR stage, yes.

MR. CROWE: Okay, okay, that does make it a bit confusing. So the primers are mandatory. You can't do PCR without them.

MR. BUSTIN: No.

MR. CROWE: But the probe is, as you say, extra insurance.

MR. BUSTIN: It's optional. And for the diagnostic assay, you would use a probe. For research purposes, you would not always use a probe because obviously, using a probe adds to the cost of the assay.

MR. CROWE: Okay. Now, one of the things you're very concerned about -- you have a 2017 paper which is mostly where I learned about some of the issues with RT PCR. And you start by talking about the crisis of replication in science, and you referred to a situation where there were five studies that were attempted to be replicated. Two were able to be replicated, one could not be, and two were uninterpretable. I wasn't exactly sure what that meant. But you seem to be saying that there's a lot of use of RT PCR that produces numbers and people use those numbers. But if you go back to try to do the same thing, you may get different numbers, different results.

MR. BUSTIN: Yes. In principle, that is correct. Depending on how you carry out the RT PCR, how you prepare your samples, how you -- which enzymes you use, which protocols you use, and how you interpret your data, you can end up with wildly different results.

MR. CROWE: Okay. So if we start to talk about the RNA extraction, you talked about co-purification of inhibitors, and I assume these are inhibitors of DNA polymerase?

MR. BUSTIN: Yes. Both reverse transcriptase and the attack polymerase using the PCR are somewhat sensitive to inhibition from product -- from compounds that are commonly present in biological samples.

MR. CROWE: Okay, so that would mean that if you don't do your RNA extraction properly or carefully, that you could end up with less DNA because you're inhibiting it through something that you took from the sample.

MR. BUSTIN: No. What happens is that you may end up with the same amount of RNA or DNA for that matter.

MR. CROWE: Right.

MR. BUSTIN: But because the enzymes are inhibited, you apparently have less than you thought you have.

MR. CROWE: Right, right.

MR. BUSTIN: There's an enzyme inhibition.

MR. CROWE: Okay. You also talk about the secondary structure of RNA. Is that kind of the curving and folding just like in proteins that RNA (ui)?

MR. BUSTIN: Yes. Most people, when they think of an RNA molecule, think of kind of a string, a linear string of RNA. And in real life, RNA, including the genome of the SARS-Covid-2, is extremely complex, has an extremely complex secondary structure with sequences quite distant coming together and forming double-stranded regions of the RNA. And this is important because obviously, when you're trying to put a primer into your reaction that will then prime your reverse transcription, if it happens to bind to a region that is extensively -- has an extensive secondary structure, then it will have difficulty getting in there and carrying out the reverse transcription set. So again, it can affect the sensitivity of the assay.

MR. CROWE: Okay. And there's no reliable way to straighten the RNA out to remove the secondary structure?

MR. BUSTIN: This is where the assay design becomes so important and that's why you can get such different results with different tests. If you and I design two different RT

PCR reactions and we placed our primers into different areas, and I happen to place mine into an area that has extensive secondary structure and you happen to place yours into an area that is in a loop structure, then your primer will be much more -- will allow more sensitive reverse transcription because you get more -- it will be easier to get into the RNA.

MR. CROWE: Right.

MR. BUSTIN: So you and I will get a different result based on the fact that mine was a poor assay where I don't detect anything and come up with a negative result, whereas you are fairly efficient and come up with a positive result, so our results are different because of that.

MR. CROWE: But I mean, I might -- is it because I know about the secondary structure in this hypothetical case or is it just that I'm lucky that I chose --

MR. BUSTIN: No. So there are programs that predict various secondary structures. They are not ideal but they're better than nothing. We published a long time ago some work on this and clearly, if you use these predictive models, then you can get better results. It doesn't always work but in general, if you're careful to find a loop area, then your assay will

be more sensitive.

MR. CROWE: Okay. You talk about the degradation if nucleic acids during preparation or storage. I mean, what types of, you know, storage could do this, freezing, like what are the things that could result in -- say if you store the sample for a year, maybe frozen or something, that could result in degradation?

MR. BUSTIN: I assume you're talking about RNA rather than DNA, right?

MR. CROWE: Yes, yes.

MR. BUSTIN: Well, the common-held view is that RNA is very unstable. So if you make RNA and freeze and thaw it too many times, it will degrade it.

MR. CROWE: Okay.

MR. BUSTIN: If you keep RNA at room temperature, it will degrade. If you heat it, it will certainly degrade. In real life, it's not as simple as that. It depends on the conditions. So I was involved with a trial in New Zealand, a murder trial, and there it turned out that RNA, as long as it's kept dry, can remain amplifiable and detectable for, you know, twenty years. So it's not -- it's not -- as always in biology, it's not a 100% yes-or-no answer. In general, you

try and make sure that you extract RNA as carefully as possible and store it as carefully as possible. But once you have a good RNA prep, then unless you heat it, keeping it in the freezer will keep it stable. It will not degrade substantially.

MR. CROWE: Okay. You referred to freezing and thawing like, presumably, you wouldn't thaw it more than once, right? You would freeze it --

MR. BUSTIN: No. What we would regularly do or what we tend to do is, when we make an RNA prep, we would aliquote it and then take the first aliquote, make CDNA from this, and store the CDNA and keep the original aliquotes frozen. And if we need to get back to that prep, then we go back to a fresh prep. So we try not to do it more than two or three times.

MR. CROWE: Okay. You talk about an RNA integrity number RIN.

MR. BUSTIN: RIN, yeah.

MR. CROWE: And you're saying this is very important, that it is above 5, I think you said. So what does that mean?

MR. BUSTIN: Well, this is a colleague who published this. Most people tend not to look -- most papers tend not to look at the integrity of their RNA. So people extract RNA

and then immediately go into a CDNA synthesis. Some people use a process which uses something called a bioanalyzer, which is an instrument that looks at the 18 and 28S RNA peaks. There's an algorithm that looks like an electrotheragram of the RNA and depending on the ratio of 28 and 18S and various other small squiggles in the electrotheragram, it comes up with an RNA integrity number. So 10 would mean the RNA is the best quality possible and for example, if we're extracting from a tissue culture, you would expect a RIN number of 10. If you extract RNA from an old, degraded sample, it might be a RIN of 1, 2, 3, or 4, and then there all kinds of shades of grey in between.

MR. CROWE: Right.

MR. BUSTIN: Now, this matters mostly if you're trying to quantify your RNA. If you're simply looking for a yes-or-no answer, obviously, if it's totally degraded, you'll get nothing. But if you're trying to just see if it's there or not, then there will be very little difference between a RIN 7 and a RIN 10, whereas if you for example have a viral load, then it could make a difference.

MR. CROWE: Yes.

MR. BUSTIN: Yeah.

MR. CROWE: And if you're trying to compare -- I mean, let's say that you're trying to compare fresh samples from today, where maybe you have a high RIN integrity, and then maybe there are some stored samples with a lower RIN, does that cause problems, if you're comparing things that have different RNA integrity levels?

MR. BUSTIN: Yes. It might if it hasn't been stored correctly and if you are going for very accurate quantification. If you're going for, is it there or not, then it is less important.

MR. CROWE: Okay. So let's move on to the reverse transcription step. So this converts the RNA to complimentary DNA and this is necessary because PCR only replicates DNA not capable of replicating RNA.

MR. BUSTIN: Contact polymrase is not very efficient at replicating RNA. It does it very badly.

MR. CROWE: Okay.

MR. BUSTIN: Yes. So that's why you have to use a special enzyme called reverse transcriptase, yes.

MR. CROWE: Right. So one of the problems you refer to is the lack of reproducibility of low copy numbers. So if there is a small amount of RNA -- if there is a small amount of RNA, you might get an unpredictable amount of complimentary

DNA. I think that's what you're saying.

MR. BUSTIN: The RT is not very efficient. It is not very efficient at converting RNA to DNA. So if you get 50% conversion, you would be happy. So if you have one copy, you may only have to detect that. If you have five copies, you may only have to detect it. Again, the problem is not so much if you're trying to see whether something is there or not, which you are -- which you tend to be with the diagnostic assays for pathogens. It is more important if you're trying to quantify accurately the amount of RNA that was there in the first place, which you would do for example for gene expression studies. Or if you're interested in very accurate viral load or fungal lode or pathogen load quantification, that's where it becomes important.

MR. CROWE: Right.

MR. BUSTIN: Just one other thing: And different reverse transcriptases have different properties and some are better than others.

MR. CROWE: Yes.

MR. BUSTIN: So that's an additional problem.

MR. CROWE: Well, you referred to a factor, I think, if I read this right, a factor of up to 100 in the -- in the

production of DNA, complimentary DNA.

MR. BUSTIN: Yes. Mica Kubister (ph), who is a Swedish scientist, published this a long time ago now, probably 15 or 16 years ago, where they showed that you can get significant differences in the amount of CDNA being produced, yes. I think RTs have become better since then and we published a few studies recently and I certainly would say that tenfold certainly is still -- is still something that can happen, yes.

MR. CROWE: Right. I mean, that seems like a really big problem if you're trying to quantify, right, if you have a tenfold difference.

MR. BUSTIN: Yes.

MR. CROWE: I mean, that's what, a couple of -- that's three PCR cycles about, right?

MR. BUSTIN: Yes. Again, as always, it's not as straightforward as that.

MR. CROWE: No.

MR. BUSTIN: Some tests -- some assays, you don't really see that, and others, you do see it. And what has been shown is that the polymerase has a preference for certain nucleotides at the three-prime end of the primers. So some primers seem to prime more efficiently than others and if

we have two assays and we just happen to have a different three-prime base, then you could get a difference based on that. So that is the kind of difficulty that you face when you generalize.

What we have recommended in our MIQE guidelines and in fact, it was recommended before that by Mikhail in one of his papers, is that you do more than one RT. See, the PCR itself is very reproducible. It's the RT that causes the problems. So what most people do is, they take RNA, do single-reverse transcription, and then they do multiple PCR reactions from that. What he recommends and what we put into the MIQE guidelines is, you should do two or three RT's because that's where the variability is, and that then gives you a measure of the uncertainty in your data.

MR. CROWE: Okay. So you do reverse transcriptase maybe three times and then you do the PCR, and you're going to get different numbers --

MR. BUSTIN: Yes.

MR. CROWE: -- from those PCR's and that kind of tells you which of your reverse transcriptase enzymes or setups -- there may be other variables -- is the most efficient.

MR. BUSTIN: Yes, but let me stress again, this is

where we're talking about quantification. And usually, when we're looking about clean expression, where people are trying to show fairly small differences for pathogens, it is less crucial because whether you have 100 or 1,000 probably doesn't make much difference as long as you can detect it reliably.

MR. CROWE: Right. We'll get to that in PCR because I have some questions about choosing the cycle number. Okay, so we've established that reverse transcription is an important step and it's got some problems with reproducibility and the amount of material that gets produced can differ quite a bit. So if we go on to the PCR step, and this is the cyclical duplication of DNA -- I mean, in theory -- let's talk about this first. In theory, if you started with one DNA strand, then on the first cycle, you would end up with two, and then it would be powers of two from then on. How close does PCR adhere to, you know, an exact doubling at each step?

MR. BUSTIN: So this is the (ui) PCR efficiency, which again, in the MIQE guidelines, the stress -- it needs to be something that's reported by authors of papers. And, again, most people don't do that. It is crucial, it is absolutely crucial because obviously, if you have -- if you double your -- the amount of targets of amplification in each cycle, you

will end up with a much greater sensitivity than if you only have a 50% efficiency. So if you want to be certain that a negative result is negative, then you need to know about the efficiency of the PCR reaction, and the efficiency of the PCR depends on lots of different things. It's fairly easy to measure and as I say, because it is an exponential amplification process, it is critical that you get as close to 100% as possible.

MR. CROWE: Right. I mean, one of the phrases I heard about PCR is that errors also multiply exponentially.

MR. BUSTIN: Yes, yes. If you have an 80% efficiency, then you have a significant difference in your end result or can have a significant difference, particularly if you're comparing two different -- two different people's assays. If mine is 100% and yours is 80%, then we have a problem in the results (ui) yes.

MR. CROWE: Right. So what I'm seeing with the Corona Virus testing is that they choose a cycle number. I've seen 36 and 37. I haven't seen it published very much.

MR. BUSTIN: Yeah.

MR. CROWE: And if you obtain sufficient DNA by that cycle, it's considered positive, and if you don't, it's considered negative.

MR. BUSTIN: Yeah.

MR. CROWE: Is there any -- I mean, that seems kind of arbitrary.

MR. BUSTIN: It's absolute nonsense, yes. It's absolute nonsense. It makes no sense whatsoever.

MR. CROWE: And another problem is that if there are quantification problems, then in effect, it's like your boundary moves, right?

MR. BUSTIN: Yes.

MR. CROWE: Like if you say had 50% efficiency in your PCR and you had 37, and you had 100% efficiency and 37, those are two completely different numbers --

MR. BUSTIN: Correct.

MR. CROWE: -- in reality.

MR. BUSTIN: Yes, yes.

MR. CROWE: Is there -- would there be a better way to determine -- because as you say, the question is really very simple. You might not care about the viral load. You want to know, does this person have this virus or whatever or not.

MR. BUSTIN: Yes.

MR. CROWE: So is there a different way to do it apart from choosing an arbitrary cycle number?

MR. BUSTIN: Okay, so let's go back to arbitrary cycle number. It depends on a lot of different things. First, different instruments give you different cycle numbers. Different PCR (ui) can give you different cycle numbers. Different lots of probes can give you different cycle numbers. So the cycle number per se is not a good measure.

The second point is that for most instruments, once you get above a cycle of about 35 of all instruments really, then you start worrying about the reliability of your result because that would be roughly equivalent to a single copy. So you would hope -- what you want do is, you want to be certain that or you want to be (ui) sure that the results you get are in the twenties to thirties. Unless you have an idea of the efficiency of your PCR and the absence of inhibition, it is very difficult to be certain of what your result represents, unless it's vaguely a bucket load of targets.

So my suggestion is and something I'm trying to establish is, if you add an RNA spike into your RNA before your reverse transcriptions, then what you would do is, you would reverse transcribe both the Corona Virus target, that's your target, plus the spike. Now because you're putting in a very -- a defined, known quantity of spike, you know what

cycle CQ to expect at the end of the run.

MR. CROWE: Right.

MR. BUSTIN: And that would depend on the particular reagent used or the instrument used. But once you establish that, it will be at least be consistent for you in your lab. It will be different in my lab.

MR. CROWE: Okay, so I might -- I might get, you know, 23 as the number I'm going to use because of my system.

MR. BUSTIN: Yes.

MR. CROWE: Somebody else might get 25. But that's okay because we have like a yardstick --

MR. BUSTIN: Correct.

MR. CROWE: -- for the different systems.

MR. BUSTIN: Yes.

MR. CROWE: But I mean, what I have seen -- go ahead.

MR. BUSTIN: Let me continue. So let's say we get a CQ -- I get 23, you get 25 for that spike. But we know if we have added -- if we haven't added sample, we would have got 20. Then we know there's some inhibition in that sample. If we get what we expect, then we can relate directly the CQ we're getting for the virus relative to the spike we've put in, and come up with a number that is now comparable between

you and between me, and that's a meaningful number.

MR. CROWE: Right, although I mean, there still are some differences, like you said there's -- based on the specific bases close to I think you said the three-prime end, there could be some differences of efficiency. So what you spiked it with and the actual virus might not behave in exactly the same way?

MR. BUSTIN: No, but it would be sufficiently accurate for the purposes of determining an approximate viral load.

MR. CROWE: Okay. If you cycle too many times, can you start to get like a ghost production of DNA?

MR. BUSTIN: Again, it depends on what your assay is. In principle, using a probe, you shouldn't. But in practice of course, you might, yes.

MR. CROWE: Yes. And so if you were to go to say 40 cycles, you might get a positive result but it might be a false positive in that your PCR has just started to string bases together.

MR. BUSTIN: I would be very unhappy about a 45 PCR.

MR. CROWE: I don't know if you know this but there's a British recommendation for Corona Virus testing that seems to indicate that every part of England can do what they like

in terms of choosing a cycle number, and they say if your cycle number is over 40, then it needs to go for further testing. But I was surprised that anybody would do that.

MR. BUSTIN: No, I think the CQ by itself -- again, we've published this. The CQ by itself is quite meaningless. You have to have other parameters that you can define before the CQ means anything.

MR. CROWE: Right. I haven't seen -- again, in the two papers I've seen that have published the CQ that they use, one defined 36 as the cutoff for positive and then I think 37 to 39 were considered indeterminate and requiring more testing. And then one used 37 as the cutoff with no indeterminate.

MR. BUSTIN: I would be very unhappy about that. This would be totally instrument and reagent and probe dependent. So yeah -- and protocol does matter. I think CQ on its own doesn't really mean an awful lot. MR. CROWE: Okay. A couple of other things that you talk about. You talk about hot-start systems, and these seem to be systems that keep the reagents warm so that when you throw samples in, there's no delay, it just starts --

MR. BUSTIN: No. Hot start means that the polymerase

is inactive at room temperature because obviously, if you throw primers and DNA or RNA together with a polymerase at room temperature, the polymerase will have some activity. And because the primer is combined nonspecifically, you might get a background based on the fact that you're getting nonspecific polymerization at room temperature.

So what they've done, many -- we're not talking about when I started this but many years ago, they developed a hot-start system where you can use either a chemical modification or an antibody that binds to the polymerase and inactivates it at room temperature. And the hot start simply means that before you do your PCR, you do a half-minute to ten-minute heating at 95 of the polymerase, and that activates it and stores the antibody or certainly allows the polymerase to have activity. And then the first cycle then starts when the kneeling (ph) temperature goes down to the correct kneeling temperature, and you reduce, significantly reduce the amount of nonspecific primer you would get.

MR. CROWE: Okay, so this is -- I guess I misinterpreted this. I thought you were saying hot start introduced problems but you're saying hot start was developed to remove these problems of polymerase activity --

MR. BUSTIN: Yeah.

MR. CROWE: -- at room temperature.

MR. BUSTIN: That's correct.

MR. CROWE: And what would polymerase activity at room temperature mean, that it's actually starting to put together a DNA string?

MR. BUSTIN: Yes. I can't remember, did I publish or not? I've got the data. What happens is, if you take two primers, DNA, and a DNA polymerase that does not have hot-start capability and just leave them on ice --

MR. CROWE: Right.

MR. BUSTIN: -- then you will get synthesis of DNA. And if you then do a PCR, you will get nonspecific amplification because the primers will have primed from sites where they normally wouldn't have bound to because the (ui) temperature -- I would say at zero degrees, the primers would bind to anything.

MR. CROWE: Okay.

MR. BUSTIN: They will bind to each other, they will bind to nonspecific DNA, so you can get a background. The problem with background always is, A, it can give a false positive but also, it can reduce the sensitivity of the assay itself.

MR. CROWE: Right. And is hot start now in widespread use?

MR. BUSTIN: Yes, I think hot start is the standard way of doing things.

MR. CROWE: Okay. You also talked about a one-step versus two-step process.

MR. BUSTIN: Yes.

MR. CROWE: I don't know if you can briefly describe --

MR. BUSTIN: That's a very fundamental distinction. I think the main difference is as follows: If you're looking to detect a pathogen, you're normally interested in taking one or two or five, up to ten different pathogens. So what you do in a one-step reaction is, the priming of the RNA to make CDNA is carried out by a specific primer, i.e. a primer that's specific for what we're interested in. So once that reaction -- in general, you mix the RT and the tack (ph) polymerase in the same tube with both forward and reverse primers. There is a method that uses a different enzyme which can do both, but we won't talk about it because it confuses the issue.

What happens then is, you give it a minute or five minutes or ten minutes at say 50 degrees, which allows your

RNA-specific primer to bind to the RNA. The reverse transcriptase then comes along and extends that primer. And after X number of minutes, you heat the whole thing to 95 degrees. This then inactivates the RT, activates the polymerase, and you start your PCR.

MR. CROWE: That sounds good.

MR. BUSTIN: This has the advantage -- it all happens in a single tube, it takes much less time, and it is fairly easy to implement. The problem is that, as we talked about RNA structure earlier, if the primers aren't well-designed, if the RNA primer particularly isn't well-designed, the assay can be not as sensitive as we might want it to be. Also, if you want to look at tens or hundreds of different targets in your sample, you may run into problems, so that's why you might use a two-step reaction.

And the difference between a one-step and a two-step is that with a two-step reaction, you can prime it with specific primers but what you tend to do is use very short, random polynucleotides. They will prime anywhere off the RNA. And you -- the first reaction then is only an RT step, where you use random priming to generate lots and lots of CDNA. And you then take an aliquote of that and put that into PCR reaction with

your PCR-specific primers. So what it means is, with the one-step reaction, you have one shot at doing your assay. With the two-step reaction, you retain your initial pool of CDNA and you can go back to that as often as you like until it's all gone, obviously.

The main advantage is that, A, you have more samples to work with. But B, the primer can be optimized for the PCR rather than for the RT and the PCR, so you can get more sensitivity. But in real life, it's not as straightforward as that. Sometimes the one-step is more sensitive, sometimes the two-step is more sensitive. It's just again something that is not predictable.

MR. CROWE: Okay. One issue I meant to talk about earlier was the length of the probes and the primers. How do you choose something that's long enough to be unique? Like, is there a problem where people use primers or probers that are either too short or too long? Do either of those cause problems?

MR. BUSTIN: Yes. Most people would use a program design (ui). So for example, I have a program which is called Beacon Designer and Alien I.D. (Ph), which allows me to design my primers. Now, most primers will be somewhere between 18

and 23 nucleotides long.

MR. CROWE: Okay.

MR. BUSTIN: The problem tends not to be the length of the primer but the fact that they haven't been designed with sufficient specificity so they can bind to other things as well. Now, with bacteria and pathogens, that tends to be less the case because you have a fairly unique sequence to target. But obviously, if you're trying to distinguish between a standard Corona Virus and the Covid-2, then you have to be very careful in designing your primers in terms of the sequence. The length itself doesn't make that much of a difference.

I think if you're trying to make the assay very, very specific and have a rather high kneeling (ph) temperature, then you would tend to have longer primers. But then again, if you're designing primers against fungal targets, for example, these are very GC-rich, so they would be much shorter than a bacterial primer, which tend to be AT-rich. For example, Clostridium Difficile, which causes diarrhea and so on, is a very rich bug. So your primers might be 25 to 28 nucleotides long because -- to get the correct kneeling temperature, whereas a fungal primer might be only 16 to 18 nucleotides long because

it is very GC-rich. So there's no general rule. There's no rule that works for everything, but in general, we try and be around about 20 nucleotides for a primer, and depending on what type of probe you use, you might use -- the probe will be somewhere between 18 and 25 nucleotides as well, although there are some specific systems that use much shorter probes.

MR. CROWE: Okay. That sounds like a highly specialized area --

MR. BUSTIN: It is.

MR. CROWE: -- that requires a lot of thought --

MR. BUSTIN: It is, it is.

MR. CROWE: -- to get it right.

MR. BUSTIN: Yes, yes. That is obviously the key issue. I've compared primers to the tires on a car. They are the thing that links the enzyme to its target. And if the primer hasn't been designed correctly, if there's any possibility of non-specificity, then that's where the whole thing goes wrong, and the probe won't help you then.

MR. CROWE: Yes, yes, okay. Let's move on to the M-I-Q-E guidelines.

MR. BUSTIN: MIQE guidelines, yeah.

MR. CROWE: MIQE, yeah, okay. That's easier to say.

So these are basically about reporting or I mean -- and you're saying -- one of your criticisms is that most people are not following the guidelines and they're not reporting enough information so that you could determine whether anything could have gone wrong with their work, or you can't reproduce it. What are the -- I guess what are the -- what was the main motivation of the MIQE guidelines and what does it mean when they don't get followed?

MR. BUSTIN: Right. Most people think that PCR is an easy thing to do because you take two primers, possibly an RT, and the PCR and that's it. A long time ago, 18 years ago, I was involved with the autism-MMR-measles controversy. You'll recall that there was some suggestion that giving the triple vaccine caused autism and that this was linked to the measles component.

MR. CROWE: Right.

MR. BUSTIN: Some of the data dad in fact, the only real data published on this used RT QPCR.

MR. CROWE: RIGHT.

MR. BUSTIN: And I was brought in to look at the data that underlined a couple of papers that were published and lots of unpublished data were presented to the court.

And it became clear to me very, very quickly that the people doing these experiments had got everything wrong they could have got wrong. Their designs were incorrect, their protocols were incorrect, the way they reported the data were incorrect, everything was wrong. So that was back in 2005. And then in 2007, we had the trial in Washington D.C., and I -- okay.

So meanwhile, we had gathered a group of people who were interested in PCR worldwide, well, Europe and the United States, and we had several meetings and we discussed real-time PCR. And it occurred to me that what we really needed was a set of guidelines that people could look to, firstly to develop their own protocols, to know what was important when designing a primer, a probe, how we extract the RNA, but secondly, also, to allow people, when they published their data, to report the things that are important for a reviewer or a reader to look at the technical quality of what these papers were reporting to see whether the results were real.

MR. CROWE: Right.

MR. BUSTIN: And also, one of the things about scientific papers is, the reason you publish is to try and let other people see what you've done so if need be, they can reproduce your results. So if you looked at papers, if

you look at papers, very often, if you have a paper that uses both -- elizer (ph) or western blotting and PCR, then the tissue culture and the elizer and the western blot are described in the greatest of detail.

MR. CROWE: Right.

MR. BUSTIN: Then there's (ui) that says, I'm going to do QPCR.

MR. CROWE: So it doesn't even give you the primers or anything like that.

MR. BUSTIN: Often, you don't get the primers and very often, they're wrong, the sequence of the primers are wrong. You don't get -- you have no idea how they do their RT, no idea how they do their PCR. And then usually -- QPCR is usually reported as a relative quantification to something and that relative quantification is more often than not incorrect as well. So that's what led us then to publish initially for PCR back in 2009, QPCR, and then for digital PCR in 2013, these MIQE guidelines. We are now discussing doing a similar sort of thing for the testing of Corona Virus.

MR. CROWE: Oh, okay. Well, that -- I think, judging from what I've seen, that might be worthwhile. So basically, the MIQE guidelines help -- tell you how you should do RT

PCR and they also tell you how you should report it so that people can evaluate what you're doing and then they can -- if they want, they could try to reproduce it as close as possible to what you actually did.

MR. BUSTIN: Yes, yes.

MR. CROWE: And you report some data that indicates that when replication of experiments occurs, you have something like a ten- to thirty-fold difference in quantification.

MR. BUSTIN: You can have, you can have, yes.

MR. CROWE: Okay. And it kind of surprises me that people would publish the wrong primers. I mean, that seems to me like kind of a secretarial job, right, just copy and paste the list of primers that you must have stored somewhere because you have to decide what they were in the first place. I don't understand how people get that wrong.

MR. BUSTIN: Yes. I think that that is probably the correct interpretation. If I was a conspiracy theorist, as some people are, then I'd say some people might publish the wrong on purpose so that people don't know what they've been doing.

MR. CROWE: Oh, that does seem a bit -- yes, that does seem a bit conspiratorial but it's like it's hard to

understand because I mean, I think if you spent a lot of time defining your primer sequence, you have to store it in a file, right? Everybody has everything stored it in a file. And you have to send it to the person who is going to generate it, and you have to make sure that every time you do this, you do it right because if you tell somebody to regenerate their own primer, you just messed up your own experiment.

MR. BUSTIN: Of course. I think one explanation is, people often get the five-prime, three-prime, three-prime five-prime orientation wrong so that primers are in the wrong orientation. That happens quite frequently.

MR. CROWE: Okay, okay.

MR. BUSTIN: I've had examples where part of the primer was left off so they only had part of the primer sequence. Why, again, I do not know. Sometimes it's a completely different primer and you have you have no idea how they came up with that particular primer but it's all kinds of means. I expect it's sloppiness rather than anything else.

MR. CROWE: Yeah, that can explain a lot of things. Okay, so let's move on to sequencing, and one of the confusions I have is, are you sequencing the RNA or the complimentary DNA?

MR. BUSTIN: In RT PCR and in QPCR, in digital PCR, we don't sequence at all.

MR. CROWE: Right.

MR. BUSTIN: There's no sequencing involved. The only thing that's involved is, you amplify your product and you use your probe to detect that product. Because you have a specific probe that binds only to the sequence or in theory binds only to the sequence that you're trying to amplify, you are convinced that you're getting the right thing, okay? Now, if you have a single RT PCR with a single probe, you get a single sequence. You get a single target that you can amplify and detect.

Sequencing is quite different because there, you can look at any RNA you like and you don't need to have a -- for PCR, you need to know what you're looking for. With sequencing, you don't need to know what you're looking for. You just generate lots and lots of sequence, which is sequence from a CDNA but that then can be read back to the RNA itself.

MR. CROWE: Right, right.

MR. BUSTIN: So you do convert your RNA into CDNA and, again, that is a critical point because, again, you've got this RT problem here. But if you're simply trying to see what messengers or what pathogens are present, then you can

do an RT PCR reaction and feed that into a sequencing reaction, and gets lots and lots of information about what is present.

MR. CROWE: Right. And you need the PCR step in order to generate enough material to do the sequencing, right?

MR. BUSTIN: Enough material, yeah. It's called a library. You prepare a library of your clear target, yes.

MR. CROWE: Okay. I think I have come to the end of my questions. I can't believe how much we got through today and I appreciate your patience with somebody who doesn't know near as much about this subject as you do. Is there anything else you'd like to add that is really important that we didn't manage to get to?

MR. BUSTIN: Well, only that I think there is a real reproducibility problem in science in general and certainly biological/biomedical science in particular, and it is something that is just not acknowledged enough. And it looks as though, you know, the editors of major journals aren't really that interested in making sure that the papers they publish are technically sound because there are so many examples now where some, you know, high-profile paper is published that then needs to be retracted. The journal gets its publicity. They are commercial enterprises and I think as long as they

can publish something that gets press attention, they're quite happy.

MR. CROWE: Well, it seems like there's a bit of a feeding frenzy right now, like I notice that it doesn't take very long to get a paper published in like the New England Journal of Medicine or JAMA or something like that. If you've got something hot on the Corona Virus, it's just a matter of a couple of days.

MR. BUSTIN: That is true as well, yes, but it also depends on who is publishing and what institution it comes from. There are lots of things that are wrong about our present publication system in biology but that's just something I would highlight, and it looks as though something like RT PCR, which everyone thinks is easy, is particularly prone to problems.

MR. CROWE: Well, okay, so just a couple of questions, sort of more philosophical questions. So if you have a nice, shiny machine and you put samples in it and the machine does everything for you and at the end, it produces a graph or a number or, you know, something like that.

MR. BUSTIN: Yes.

MR. CROWE: Does that lead you to believe that it's

simpler and more precise than it might actually be?

MR. BUSTIN: Yes, absolutely.

MR. CROWE: Okay. The second things is, we l8ive in a digital world. Since the 1950's, we've had computers and everyone knows that computers are binary. And really, the only thing in biology that is digital that I can think of is DNA and RNA, in that it is a code of four different bases. And so, you know, if you have the sequence of DNA or RNA in a computer, you can generate that RNA or DNA, whereas you probably couldn't do that for a protein --

MR. BUSTIN: Yes.

MR. CROWE: -- because of the confirmation and things like that. So that does lead us to believe that we have to focus on RNA and DNA and ignore all the uncertainties around the actual manipulation of RNA and DNA, which is very much chemical and biological?

MR. BUSTIN: Yes, it has been in the past certainly. I think it has got better but I think we used to do RNA quantification because we could do it. We couldn't easily do protein quantification so that was certainly something. Nowadays, I think most papers or any good journal would require not just an RNA-based result but also some kind of protein validation.

It is slightly different of course for pathogen papers because there you have an RNA so in order to look at gene expression, you look at the presence and actions of a pathogen. But if you're trying to do studies on the biology of a virus, then you definitely have to have both RNA and DNA data.

Also bear in mind, we used to think there's mRNA, ribosome RNA, and transfer RNA. Now we know there's all kinds of other RNA's, between antisence and large nucleus -- large, small, and micro, and god knows what else. So it is extremely complex and having a single RT QPCR test to detect something is great but it needs to be put in the context of a whole lot of other experiments that are carried out to validate and perhaps explain what the result is. So unfortunately, that means you have to do things much more slowly than we are given. We are all under pressure to publish and get results out, and it doesn't help that we have -- that some of our results then tend to be incorrect.

MR. CROWE: Yes, yes, agreed. I think we live in a society where speed is sometimes the most important parameter over accuracy and things like that.

MR. BUSTIN: Yes.

MR. CROWE: I would just like to thank you for taking

a considerable amount of time to discuss this issue, which I think is very important in the modern world.

MR. BUSTIN: It's a pleasure. Thank you very much for asking me to (ui) I could explain some things to you, and I hope it helps somebody understand a little bit more about the current problems we're seeing with testing, which are considerable, but a lot of them are self-imposed, particularly in the U.K. I'm not sure what's happening in Canada but I see in the United States as well, it just is mind-boggling how we ended up in a situation like this.

MR. CROWE: Yes, yes, there's many confusions about this whole thing, and I think everybody is off doing their own thing.

MR. BUSTIN: But what I find particularly amazing -- I don't know what it's like with you but people refer to PCR antigen test. So our government ministers talk about antigen tests, our BBC reporters -- antigen tests. It has nothing to do with antigen tests.

MR. CROWE: Okay.

MR. BUSTIN: It's a lack of understanding.

MR. CROWE: One more question. Maybe you know this. Avid announced that they had a five-minute, molecular test

for the Corona Virus. Do you have any idea what that might be? It can't be a PCR, right, like five minutes is just impossible.

MR. BUSTIN: Well, it's not impossible but it could be a lamp, so isothermal, or it could be a lateral flow device, so protein-based.

MR. CROWE: Okay.

MR. BUSTIN: If you have the antigen bound to a lateral flow device, put a drop of blood on it, and if there's antibodies present, that could be detected in five minutes.

MR. CROWE: Right, but it said a molecular test so to me, that sounded like -- I don't know what that means because everything is a molecule.

MR. BUSTIN: Exactly. Is it a nasal swab they're using or is it a blood sample, do you know?

MR. CROWE: I think it would be a nasal swab --

MR. BUSTIN: Okay.

MR. CROWE: --because it was specific for the domestic violence.

MR. BUSTIN: Okay, it could be a lamp, which is an isothermal application, which possibly could work in five minutes.

MR. CROWE: Okay. Well, maybe one day, we'll find

out.

MR. BUSTIN: I'm sure, yes.

MR. CROWE: I haven't gotten any technical information on that. Well, thank you so much for joining me today. I really do appreciate it.

MR. BUSTIN: It's a pleasure. Thank you, cheers.