Steve Lewis 00:00
Welcome to Speaking of Mol Bio, a podcast series about molecular biology and its trending applications in life sciences. I'm your host. Steve Lewis, bringing you another Mol Bio Minutes mini episode. Today, we feature Thermo Fisher senior sales training specialist, Dr. Will Barnes, sharing his insights on cDNA innovation and how he has used Thermo Fisher technologies in his plant biology research. I think this is one you'll enjoy. And finally, be sure to stay tuned until the end of the episode for something new we're trying here. It's a special promotional offer exclusively for Speaking of Mol Bio listeners. That means you won't want to miss this limited time promotion, so stick around until my closing remarks to get the details.

William Barnes, PhD 01:00
Thank you, Steve, very happy to be joining you today. So a bit about my background. I'm actually a plant biologist by training, but I started off as a chemist. As chemistry really became more of a quantitative focus and more related to physics, I was looking for some sort of application in in the life sciences. Luckily, as an undergrad at Cornell, I was able to find plenty of application both in the medical fields and plant biology, but the interest in biofuels and using plants to create biofuels specifically won me over in terms of my research interest. This connects to my current role as a sales trainer in molecular biology with Thermo Fisher in that in the plant biology space there are nowhere near as many molecular tools as there are for, say, mammalian or medical biology. So from the ground up, using very foundational molecular biology practices, I became very familiar with molecular biology, the history of it and the prominence and importance of it in modern research.

As a burgeoning molecular biologist, one of the most significant lessons throughout my time at the bench that continues to be more and more apparent today, even in my role, time is the most valuable thing in research and also the most expensive thing. So, it's really important to advocate for using the best technology to accomplish your goals, to provide the best data, give you the most confidence and allow you to increase productivity while using your resources wisely. Often, when we think about plant molecular biology, our minds will immediately go to genetically modified organisms. Which is fantastic and has a very prominent space in the future and our current sectors such as food security and even things like pharmaceuticals, but the way that I used molecular biology was a little bit different in that I was investigating the basic mechanisms of how plants built themselves. In my need, I really needed to focus on cloning genes related to cell wall synthesis, which tend to be very large, genes up to 6kb, which can provide some unique challenges. As I was doing this, I was essentially trying to study protein-protein interactions, generate fusion proteins and to perform expression analyses. So I've really used molecular biology tools for a lot of applications. But behind these needs, I really needed the tools to generate cDNA with high fidelity from a difficult collection of samples. And as I transitioned into my PhD at Penn State, I found some additional challenges in that I was joining a new lab where startup funding didn't allow some of the current tools that we have available, like gene synthesis, so all of the core molecular biology work for whatever I needed was on me and what I could accomplish at the bench.

As molecular biologists, we're very familiar with the central dogma in that we have DNA encoding genes that are transcribed into mRNA molecules that are then translated into proteins. A lot of my work focused on the RNA section of that, that middle transcription step. And RNA is thought of as one of the

most difficult macromolecules to work with, even though they all certainly have their caveats and challenges due to its transience and inherent instability across all applications, whether that's next generation sequencing, cloning, in my case, and even expression studies. So one of my main goals and needs as a researcher was to use high performance RNA reagents to ensure the integrity, accuracy and reliability of RNA. As far as what I did with that RNA, once it was in my hands, whether it was cloning or expression analysis, I followed the same basic workflow related to RNA. I would extract total RNA, degrade the genomic DNA, wash the RNA, elute it, and then quantify to see exactly how much I had and qualify it as well, to see the quality of that RNA, which was often a pain point. And then finally, when I had sufficient RNA of high enough quality, I would synthesize cDNA and then jump right into my cloning workflow, where I exercised the full repertoire of molecular techniques, from restriction enzymes to Gateway cloning and Gibson assembly. So really going from those foundational single insert clones to higher level arrangements of multiple fragments.

As a plant biologist, one of the major challenges in generating high quality cDNA was that during the cloning steps, I would often go through my workflow and then find out at the sequencing stage that I would have aberrant sequences, truncated cDNAs, or the inability to amplify full coding sequences, likely because of how massive these plant genes can be, but also because they contain repetitive elements, or sometimes I would hit the wrong target, because plants historically have undergone several whole genome duplication events to where there are many copies of similar genes, which can make targeting exactly your gene a little difficult. So these issues are likely associated with those early stages of sample preparation and cDNA synthesis. And you don't want to find out that you have problems at the very end, like I so often did. So to deal with these difficult sequences that repeat or are truncated or have difficult secondary structures, sometimes I had to get a little creative with my reverse transcription and subsequent cloning workflows. Beyond cloning, if I was performing something to determine the expression of genes such as qPCR, I would often receive inconsistent results that I would verify on a gel, whether it was smeary bands, bands of different sizes, highly irregular and variable qPCR results. And despite accounting for transcript variants and all the things that you can check on, these problems kept coming back and interfering with my work.

So as I was in grad school and really diving into these issues that became personal to me, I wish that I had taken the time to step back and see what else I could do, what other tools were really out there. So often, when you're just trying to get that data, you lose sight of your options, and possibly better ways to do so. So, although I was interested in just getting a gene cloned or finding out the transcript abundance of a certain target, I wasn't always thinking about the sample prep, the reverse transcription that really determines how well you might expect to detect those things. And that's kind of where my interest and my reliance, for now 15 years, on Thermo Fisher products, has really come in. I found that with a lot of banging my head against the wall and troubleshooting, that a lot of my major considerations and issues were linked to that initial sample prep. So not only RNA isolation, but maintain the integrity of that RNA, and then being able to convert that RNA into an excellent cDNA resource. In my line of work, I use cDNA as not just a library for sequencing, but really a library for me to continue to go back to. So, I relied on the same RNA preparation and the same cDNA synthesis reaction for everything from cloning the simplest things, to those hierarchical cloning techniques such as Gateway recombination and Gibson assembly, but then also for expression analysis. So, I'm asking

a lot of my samples, and as such, I should have taken the time and I had the wherewithal to really care about how they were being prepared and using the best reagents to do so.

So some of the issues that I found to be repeatedly coming up throughout my workflows, throughout many years of the bench, especially with my plant samples, was that I was getting a lot of carbohydrate contamination. Now that might be expected because of the tissues I was working with which contain a lot of starch, which is essentially glucose, or the cell walls, which contain many different sugars and all different linkages that could easily be released during these common techniques that we use to isolate nucleic acids, because they're both large, charged, for the most part, macromolecules. Additionally, when I was working with cell wall mutants, it made the cell walls even more fragile and allowed more of these contaminants to enter my samples. As I was going through this over and over again and continually using the NanoDrop and seeing those poor 260 nm to 230 nm ratios and everything indicating that my samples weren't good enough, I really wanted to look into some different areas to find what I could do to improve my workflows. So in doing so, I would often use a neighboring labs Qubit instrument as an alternative to NanoDrop, because the Qubit can specifically detect RNA or DNA from complex mixtures. It could also indicate the quality of your RNA with the RNA IQ assays. And that here, I was really starting to understand exactly what was going on, rather than just not seeing that my sample was good enough. In my first introduction of some of Thermo's analytical tools, it was already giving me more insight into my research on the sample prep side of things, TRIzol, which I'm sure everyone is familiar with, is a historical go-to product, and it's equally as effective in plants, at least for most tissues. But I also found that when isolating RNA, if you went to really tough tissues that have a lot of phenolics, such as roots or using flowers, which contain a lot of pigments, those molecules can easily impact your recovery and the quality of your RNA sample. So here I started to explore some different technologies, such as the GeneJET column-based kits for plants, and in special applications, like when I was an undergrad isolating RNA from the moss *Physcomitrella patens*, we found that magnetic bead technology was really the best way to get the most out of our sample.

So, it's interesting how I was now starting to learn how different sample preparation methods aren't created equal and can really have a prominent impact on your results. And I should mention that this issue was not unique to myself. I had collaborators and colleagues in my lab using different model species, from mosses to grasses, that all had these unique needs. So one more mention of a unique need was a colleague of mine was studying *Brachypodium* and similarly generating these mutant lines. And when we were trying to verify those mutant lines collectively as a lab, because it was such a large effort, we were having a lot of trouble just doing the simple work of isolating genomic DNA and doing quick PCR reactions on it. Essentially, we're seeing the same thing where, although not in an RNA workflow, our DNA workflows weren't even proper for all samples. And in this case, we actually found that using some of Thermo Fisher's 1-Step PCR kits was the most certain way to get solid PCR results from our *Brachypodium* grass cell wall samples.

So often in lab, when you have a bunch of colleagues all working on the same problem, and everyone's having their own unique struggles and having ideas. The old adage kept coming up that, “The solution to pollution was dilution,” which essentially just dilutes out all the inhibitors that are giving you downstream problems, but it's also diluting out your sample to where you need more sensitive reagents, or you might be diluting out of the range of detection. So although it might help in some

cases, in lower yielding samples or particularly dirty samples, we're not going to have any luck of diluting. So in this case, we had to recruit the use of some really sensitive reagents, such as the Superscript IV VILO Reverse Transcriptase Master Mix. This basically allowed us to create cDNA libraries from samples that weren't the cleanest, but also, if our yield wasn't the highest in the midst of this kind of dirty mixture, the Superscript IV VILO mix could overcome that and still produce high quality cDNA. Additionally, the VILO mix provides a mixture of oligo dT primers and random hexamers, which provide really full coverage of our transcriptome that we were essentially trying to probe. But I should say that even with VILO's excellent technology, and really giving us the foundation to build upon for our RNA workflows, as we created cDNA, we are still having some issues that could be related to primers. It might have been better in those cases to include both the oligo dT and random hexamer primers, but even some gene specific primers for our particular targets. One more thing that I'd like to point out about the Superscript IV VILO Master Mix that really helped us, because I do feel indebted to it, is that because these plant RNAs are so long and have such significant secondary structure, we also really relied on the thermal tolerance of Superscript IV just being able to melt the secondary structures of our RNA molecules for better conversion to cDNA, which really allowed us to clone these huge transcripts.

Nowadays, if I had to go back into the lab and get my hands dirty again and generate these samples, I would love to get some insight from the new NanoDrop Ultra where it's actually detecting and giving you some insight into sources of contamination, and to continue to use the Qubit for specificity. And even on the sample prep side of things, we were constantly seeing genomic DNA contamination, which likely indicated that our DNase treatments just were not sufficient. This makes sense if you've ever done the little at home experiment where you isolate DNA from a strawberry. And strawberries are highly polyploid, so there are many, many copies of the genome to where you can physically see the DNA, although that isn't true for most plants, plant genomes can be large and very difficult to degrade. Using some of thermo Fisher's DNA solutions, like the Turbo DNase or the DNA-free DNA Removal Kit, really would have Increased our efficiency of DNA removal and given us cleaner RNA samples as well. Because there was nothing worse than doing a PCR, or doing some sequencing and finding out that you actually amplified an even larger genomic DNA sequence with some aberrant characteristics, compared to the coding sequence that you wanted. And additionally, using reagents such as the DNA-free DNA Removal Kit, not only is going to provide you the fantastic results of completely degrading that DNA, but it actually saves a lot of time on the sample prep side of things too. Where instead of throwing your RNA sample back on a column, going through the washes, probably losing a decent amount of that sample on the column, the DNA-free DNA Removal Kit actually contains some beads that are referred to as the DNase inactivation reagent that essentially just sequester that degraded DNA and leave your RNA samples in suspension. So not only would I be using a better solution, it would actually be saving me time, not just in the long run based on results, but even at the step-by-step basis, where I'm just trying to prepare the sample, trying to purify my sample, trying to keep my RNA at high quality and to convert that to cDNA. Additionally, I mentioned before that plants have a lot of duplicated genes and are pretty large gene families. For example, one of the gene families that I studied during my PhD had 68 members, which is quite large, and there's a decent amount of sequence homology between some of those genes. In that way, I was often using SYBR Green for my qPCR readout, which is not specific and really just detects amplicons. But if anything is amplifying, you really can't tell exactly what that is, you're just relying on the specificity of your primers. However, as I joined Thermo Fisher and started supporting some of these products on the tech support team, the TaqMan assays were

immediately ringing about as this would have been a great way to really increase the specificity when investigating complex gene families or sequences that might be relatively common in your sample.

So in closing, and looking back fondly at my time at the bench, I wish that I wouldn't have been so stubborn and only thought about the price of new reagents or just what we had available in the freezer, when my greatest need as a student was the expertise and understanding of how to really optimize success, not just towards that final piece of data, but at each step of any complicated workflow. Sometimes you get tunnel vision and hyper focus on the results, but you really need to think about how you generate the data. All in all, it's not always possible to rely on being resourceful, especially as a student, and sometimes you do need the better solution, expertise and a bit of help, all of which I've found at Thermo Fisher.

Steve Lewis 18:12
That was Dr. Will Barnes, senior sales training specialist at Thermo Fisher Scientific. As always, for these Mol Bio Minutes mini episodes, we recommend that you check out the Episode Notes to find links to the helpful resources that Will covered today. And now for being a loyal listener, for the first time ever on the podcast, we have a special promotional offer. Be one of the first 75 listeners to spend $500 and get 40% off all eligible reverse transcriptase, plastics, enzymes and cloning reagents. If you are in the United States, enter promo code C, A, Z, D, U, A, when you place an eligible order. For customers in Canada, please use promo code C,Z, 7, F, 1, 9. This promotion is only available until October 27th so act fast. Check out the Episode Notes for a direct link or visit thermofisher.com/sombpromo for more details, that's thermofisher.com/ S O M B promo. Terms and conditions apply.