Steve Lewis 00:10
Welcome to Speaking of Mol Bio, a podcast series about molecular biology and its trending applications in life sciences. I'm Steve Lewis, bringing you another episode of our Mol Bio Minutes mini episodes. Today's topic is PCR accuracy. Laurynas Alijošius returns to the show with more insights and advice on the topic.

Laurynas Alijošius 00:35
Hello everyone, and welcome back to our podcast. Today we're going to start with a little story, so let's fly right into it. So some time ago, I wanted to make some specific DNA to use it for my model system for my experiments. I did some PCR from a biological template, then I cloned amplified DNA, and then I sequenced it. But it turned out that my DNA was wrong. It had multiple errors in this sequence, and I don't know, I was thinking, "What should I do, then?" I was thinking like, "Should I replace all the wrong nucleotides one by one, or should I just redo all the experiments from scratch and expect that I will get better results on next time?" I'm not going to lie here. It was frustrating, but luckily for all of us we can reduce all these issues that occurred by boosting the accuracy of the PCR reaction. So that's what we're going to talk about today. We're going to talk about today about the accuracy of the polymerase chain reaction and everything that comes along with it.

First things first. Let's talk about what are the benefits of having an accurate PCR. Accuracy suggests that there will be less errors introduced during your PCR reaction, so automatically, we'll have a higher confidence in your results. And that's great, right? Having low numbers of errors is important for some methods. Let's take, for example, sequencing. It's very important to have the correct amplified sequence for sequencing experiments such as de novo sequencing or if you're sequencing your samples after you perform mutagenesis experiments. But this can also be important for simple experiments, such as just cloning your plasmid construct and analyzing if it was cloned correctly and everything went smoothly. Because having accurate PCR will just increase the chance that you will get your right sequence on the first try. There's other benefits as well. Let's say if you're using accurate DNA polymerases, you can also expand your PCR abilities in general. You can make them both short and long PCR products. So let's say if you use conventional *taq* DNA polymerase, the *taq* DNA polymerase can amplify DNA products up to three to five kilobases in length. On the other hand, if you would use an accurate, high fidelity, advanced polymerase, then you can produce PCR products up to 20 kilobases in length, or even longer products. This is very helpful if you work with large genes, or if you would like to prepare some long-read sequencing libraries that could help you a lot. And if your PCR is accurate, like I said earlier, your results would be more reliable. So in that sense, you will need to repeat your experiments less times. So that means that you will use less consumables. You will use less reagents, in general, you will use less money, and of course, you will save more of your precious, precious time. So remember the story I told you at the beginning, if I would have used accurate PCR back then, I would have saved myself so much time and frustration. It would have been so much easier.

Honestly, now we know why it's nice to have good PCR, right? But what about the roots of the accuracy of the PCR reaction? I mean, why does the PCR reaction accuracy can sometimes be low, and what can cause this low accuracy thing? There can be several factors. So let's see what's up one by one. As I suggested before, one of the major factors here for the PCR accuracy is the fidelity of your main

enzyme, that is DNA polymerase. Fidelity is like the thing that we call the accuracy of the, of our DNA polymerase. So for example, if we use the conventional DNA polymerases, like *taq* DNA polymerase, they tend to use and have, like lower fidelity levels, because it lacks proofreading activity that can also be called five prime to three prime proofreading exonuclease activity. If the polymerase lacks this activity, it cannot fix its own amplification errors that is makes during the DNA synthesis. PCR accuracy can also be impacted by other components of your PCR reaction. So for example, if your primer design is a bit poorer, that can lead to some unwanted processes like formation of primer dimers, or primers binding to the template in a non-specific fashion, or just primers can be just too short, and that would mean that they could bind to some non-specific locations on your DNA template and wrong sequence could be amplified. So, if you would like to avoid all this, in the notes of this episode, you can find tools such for such applications like primer designing or just to calculate the temperature of your primer melting conditions. These tools could help you with this primer designing and planning.

The quality of another important components is also important, and that important component is the DNA template. So if there are impurities in your DNA template sample, let's say some leftovers from the purification reagents, a phenol or salts, all of these leftovers and impurities, they can impact the quality of your PCR in a negative way. Your PCR can also be compromised if your DNA sample is contaminated with some foreign DNA, so that could mess up your general PCR results pool or, or, let's say, if your DNA template is modified or degraded. For example, if you use DNA from the FFPE samples, that stands for the formalin fixed paraffin embedded samples. DNA originating from the FFPE, for example, it tends to be degraded in a way, so that can cause some problems.

Also, the overall composition of your PCR reaction mix can impact the accuracy of the PCR. For example, let's take nucleotides. Nucleotides are like bricks or like building blocks for the newly synthesized DNA strand. So if your bricks are of lower quality, then your house will be of not so good quality too, right. So, the same goes for the PCR, and the accuracy and the quality of your PCR product could be also compromised a bit here. Other components, such as ions, let's say magnesium ions. Magnesium ions are essential for your PCR reaction to function, to happen. But if you have too high a concentration of magnesium ions, that can negatively affect the accuracy. And there are some other ions that are called similarly, but not the same. These are manganese ions. Oh, you want to avoid these manganese ions, because they can increase and mess up your polymerase error rate in a bad way. We want to avoid that.

So we figured out that components impact the accuracy of the PCR, right. But what about the cycling conditions? Does it also affect the accuracy of the PCR or not? Well, the answer right here is yes, suboptimal cycling conditions can also lead to inaccurate PCR, unfortunately. So for example, if you use very long denaturation times that can increase the likelihood of non-specific binding and eventually lead to some amplification errors, or if you perform PCR too many times, I mean, if you perform too many cycles for your PCR, that can also result in things like accumulation of errors, but also there will be a higher chance of non-specific amplifications in the later cycles if you use too many cycles for your PCR. And another thing to be mindful of is to avoid using low annealing temperatures for your primer annealing. Because that can increase the chances that your primers will bind nonspecifically and eventually that could lead to some non-specific amplification too. As you can see, there's many factors that you can go into detail and that can affect the accuracy of your PCR.

So you know what can make the accuracy go low, right. But what can we do to improve all this? So okay, okay, so the best is to address these factors one by one. Let's in the same fashion that we talked about earlier. So let's say your DNA polymerase is low fidelity type, or your polymerase doesn't have the proofreading activity that could fix errors. I mean, well, you can change that just by simply changing the polymerase that you use for your PCR reaction. So for example, you could change *taq* DNA polymerase to advanced polymerase, let's say some Platinum SuperFi DNA polymerase, which is excellent, high fidelity and is like more than 300 times more precise than the conventional *taq* polymerase. Difference here is huge. Difference is huge. And also, if you use a polymerase with a hot start feature, you can ensure then that your PCR will start only under your controled condition, and that would decrease the chance that some non-specific amplification would happen in your PCR, at least in the beginning of the PCR reaction. If all this sounds intriguing and interesting, you can use the link in the notes of this episode and learn more about how these polymerases work and learn more about this fidelity in general.

Let's go back to the PCR components. So what about the other components? If we're talking about other components, rather than the DNA polymerase, we want to have the highest quality possible for the highest PCR accuracy, always follow guidelines such as primer design recommendations to have the best primers for your PCR, and for that, you can use our resources pages, or you can follow the manuals of polymerases or the PCR kits. Also for DNA template, you could use a purified DNA template when possible. Otherwise, if you need to use things like crude samples for a template, or you have to deal with impurities in your DNA template, then you could consider using an DNA polymerase that is resistant to inhibitors so that could help you, in this case, a lot. For your reaction mix, the most convenient option, we believe, is what we love to do in our lab is to use ready to use PCR master mixes or buffers, because in that case, we have an optimal reaction mix composition and we are avoiding things like additional pipetting errors that we don't need, or we lower the risk of the contamination of our samples. And it also, we just save some extra time for coffee or for some chatting, so that's nice, so just to go home earlier. In other cases, if you need and choose to use and make your own PCR mix composition, to optimize it, you can follow the guidelines for components such as magnesium ions or nucleotides or primer concentrations for the PCR. Just remember to avoid these manganese ions that I mentioned earlier, because they can mess up the accuracy of the PCR.

Laurynas Alijošius 15:07
So now that we got the PCR components ready, let's move on to the cycling conditions for the accurate PCR. Starting with denaturation, try to use standard times according to your DNA polymerase or your master mix or the sample type that you're using, because for things like crude samples, you may need a bit longer denaturation time. But as I mentioned earlier, you don't want to have a very long denaturation time because that can cause some accuracy problems. But can also optimize other steps of PCR as well. Let's say the primer annealing step, the temperature of this step. You need to align the temperature with your design primers to ensure that your primers will bind specifically and they will not provide some non-specific amplification. And finally, for the whole PCR program, try to limit the number of overall PCR cycles to the minimum. I would say a good starting point here is like 30 cycles. Start with 30 cycles and then test your PCR product, see if it's good and if it's a good product, then you can get that number of PCR cycles to even lower level, and just test it out and see if it works, if the yields are

enough and things like that. So the principle here is simple. Having less PCR cycles means that there will be less amplification errors that will be reintroduced and multiplied in the following cycles of the PCR.

If you want to learn more about these proper cycling conditions, you can find the link in the notes to a page that outlines PCR cycling tips to help you optimize it. So that's right about it for the starters of your accurate PCR journey. If you follow all these recommendations that we talked about here, you will achieve PCR results with higher confidence, and all that means that less time there will be for a scientific head scratcher. And there will be a lower need to repeat your experiments, but most importantly, you will have more joyful time for your favorite hobbies or just any other stuff that your dreams are made of. So thanks for tuning in. We are flying out. Feel free to dig more in the notes of the episode. Until the next time happy experimenting and may your PCR accuracy reach high to turn out sublime.

Steve Lewis 17:52
That was Laurynas Alijošius, Scientist III at Thermo Fisher Scientific. As always, for these Mol Bio Minutes episodes, we recommend that you check out the Episode Notes to find links to the helpful resources that Laurynas has covered today. We'll have another Mol Bio Minutes episode next month, but up before that is a great interview and discussion I had about some amazing science. 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.