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. Our friend and colleague, Laurynas Alijošius, is back again today, this time with insights on RCA, rolling circle amplification, and its use with next gen sequencing.

Laurynas Alijošius 00:40
Hello everyone, and welcome to the new podcast episode. Today, I would like to start with a little story. So, once upon a time, in a lab not so far away, I wanted to sequence and analyze the circular DNA genomes of some viruses, but I had a problem. The amounts of the extracted DNA material were always very low, so I couldn't prepare a library for the next generation sequencing of these viral genomes. So that got me thinking, "What can I do? Maybe I can amplify this DNA with PCR." But to make matters worse, I couldn't even perform PCR to amplify the DNA because I had no primers for this unknown sequence. So it seemed like this was the end for this experiment. This was the end of the road. But luckily, one of my colleagues knew how to solve this problem. He knew just the right method to amplify very low amounts of circular DNA, and that method is rolling circle amplification.

So what kind of method is the rolling circle amplification? Or we can just call it, shortly as RCA. To see the whole picture, imagine RCA method as one piece of the delicious pie made up of different amplification methods. RCA is one type of multiple displacement amplification. Multiple displacement amplification, or can be called shortly as MDA, is the general term for a type of isothermal amplification where strand displacement occurs. Oh, so now we're going down the rabbit hole all these terms right here, RCA, MDA, isothermal amplification. So, it gets kind of confusing, right? So what is isothermal amplification, then? And is it something, some amplification like PCR? Well, not exactly. Isothermal amplification is a bit different from PCR. It's more like a chill cousin of the PCR. The difference is that it works perfectly at a single temperature. And unlike the PCR, when it's performed it is then running amplification many cycles with temperatures being changed constantly and so on. Isothermal amplification does not need that. It runs at a single temperature. What does this strand displacement mean? Then picture this, during multiple displacement amplification reaction, the polymerase is like a bulldozer pushing newly synthesized DNA strands out of the way to create super long strands of DNA. It does sound a bit confusing, right?

So, let's break it down step by step. First, short random primers bind to many different locations on the DNA template. Then the polymerase comes in, binds and performs DNA synthesis. When the polymerase encounters another new DNA strand it pushes that strand other way and continues with a synthesis. In the result, you get very long stretches of branched DNA, and in that way, you get huge amounts of DNA that can be amplified from a small amount of template. So both RCA and whole genome amplification are part of the MDA family. Whole genome amplification is used when amplification occurs from linear templates, such as linear genomic DNA, while RCA is used for circular DNA templates, such as plasmids, viral genomes, or ligated DNA molecules. Think of the circular DNA genomes of the viruses I was working with, and I told you in the story previously, so that could be a good example. And so RCA works in a similar fashion as we described MDA earlier. The polymerase rolls and rolls around the circular DNA template, then pushes out other DNA strands in its path and

keeps rolling around the circular DNA using it as a never-ending template, and ultimately it produces a very long and branched DNA product.

So now that we familiarized ourselves with some terms, let's dig deeper and uncover the magic of the RCA method. So what makes RCA so special compared to PCR? Well, first off, RCA uses a constant amplification temperature, so there's no need to program multiple cycles with different temperatures and step durations, plus it operates at a much lower temperature than PCR, so it makes it super easy to perform amplification with a simple thermal cycler or just a thermal block. Another cool thing about RCA is that you don't need to design and use primer specific to the template. This means you can amplify the DNA template without even knowing the sequence. I mean, how awesome is that? It makes things so much easier. But what is the main element of the RCA method. It's the DNA polymerase, and usually it's a Bst or phi29 DNA polymerase that are used for RCA reaction. However, phi29 DNA polymerase is much more popular, and it is the whole crowd's favorite here, because it has proofreading activity and that makes it a high-fidelity polymerase.

What other features make the phi29 DNA polymerase work for RCA? Firstly, it's a strong strand displacement activity that allows the polymerase to push DNA strands that are in the way while synthesizing a new one. And another feature is high processivity, which enables the polymerase to create long stretches of DNA stretching even up to 70 kilobases in length. It's crazy long product. On top of that, RCA method is convenient to combine it with next generation sequencing applications because phi29 polymerase has low GC content bias. Also it is a high-fidelity polymerase which is improved by the proofreading activity because of its 3-prime to 5-prime exonuclease domain. However, the wild type phi29 polymerase is not totally perfect, actually. It has a bit of GC content bias and is a bit slow, taking up to 16 hours to amplify the template. So it's kind of a long amplification process, right? It's kind of a long reaction to wait for. But don't worry, because the future is already here, and the future is engineered phi29 DNA polymerases, such as EquiPhi29 DNA polymerase. So this EquiPhi29 polymerase offers lower GC content bias. It also offers higher sensitivity and higher yields. It can produce significant yields in scale of micrograms, just from one femtogram of sample. It's very sensitive, and on top of that, it's much, much faster than the wild type phi29 DNA polymerase, because it can get the job done in just one or two hours compared to the wild type phi29 16-hour marathon. So we're saving a lot of time here with engineered polymerases. So that's about the magic of the RCA method. If you want to learn more all about this, please check out the resources of this episode for more information.

So we talked about unique RCA features, but why can it be beneficial to pair it with next generation sequencing applications? Well, sometimes the amount of your DNA material can be too low to prepare a good quality NGS library. This is where highly sensitive and high-yielding RCA or whole genome amplification can come to the rescue. They can help you amplify DNA from very low amounts of DNA or even from a single cell sample. So when it comes to combining RCA and NGS, RCA is used before the NGS library preparation step. It is used for the amplification of your starting DNA material.

What are the things to consider when running RCA before NGS? Firstly, let's talk about primer selection. For RCA and WGA, exonuclease resistant primers are needed due to the polymerases 3-prime to 5-prime exonuclease activity and usually short random primers are used for amplification,

which bind to many different locations on the template. However, if you want, you can also use specific primers instead for your experiment. And using specific primer may help you down the road. It may help to improve the number of NGS reads for your specific target of interest. There can be multiple strategies to apply this. You can perform RCA only with a specific primer, or you can try to mix the specific primer together with random primers. Or you can even use a set of specific primers. So, as you can see, there are many ways to experiment here to get the best results that you seek.

Also, there are things to consider after the amplification with RCA method. Firstly, it's post amplification debranching. RCA produces a branch DNA product and that sometimes can be disruptive for downstream applications, especially for nanopore-based sequencing. But don't worry, because there are ways to remove the branch DNA chains. For that, you can use S1 nucleus or T7 Endonuclease I and then there's post amplification cleanup as well. So clean DNA is often needed, and it's important to prepare an NGS library without disruptions. But no worries here as wel; RCA products can be purified quite conveniently, either with magnetic beads or using an affinity-based spin column.

So, we covered the basics of how RCA can be used together with NGS applications. But what are the actual cases of RCA being used together with NGS? Let's see. So, RCA is often used to help in viral genomics research, especially with low abundance or difficult samples. RCA works well with circular DNA genomes as a template, or a template can be ligated into a circular DNA form and then amplified by RCA. For example, even RNA viruses can be tackled. First, copy DNA synthesized by reverse transcription from viral RNA and then it is ligated into a circular template for the RCA reaction. Then, the successful NGS can help you to find, characterize, and identify new viral genotypes. RCA can also help when NGS is used to detect single molecule mutations. In this case, RCA-based target enrichment can be used before library preparation to improve NGS based rare variant detection. If we talk about whole genome amplification, which uses the same polymerase as RCA, it can be used similarly for single cell genomics or even for transcriptome profiling of low abundance RNA molecules, such as circular RNA. So as you can see, there are many applications where RCA or WGA can help you in improving your NGS library quality. However, RCA and WGA applications are not limited only to the product amplification or downstream NGS applications. So even more, it can be used for many things. For example, RCA can also be applied for DNA nanopore sequencing. It can be used for in vitro transcription, or cell-free protein expression, or to detect single nucleotide polymorphisms. And if you talk about whole genome amplification, it can also be applied for real time target detection. So if you are curious to learn more about these other applications, please check out the resources linked in this episode for more information.

So that's right about it, about the role of RCA in NGS. As we discussed at the beginning, if you have very low amounts of DNA template that are too low for your NGS library preparation, there is no need to worry. RCA and WGA are here to help you. Powered by the highly sensitive and highly yielding phi29 type DNA polymerases, you can improve the quality of your NGS libraries and expect better results without any headaches. And don't forget to check out and access the helpful resources in the Episode Notes. Until the next time, and may all your experiments have great results.

Steve Lewis 15:25
That was Laurynas Alijošius, Scientist III 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 Laurynas covered today. We'll have more Mol Bio Minutes episodes coming in the new year, as well as some engaging conversations about some amazing science. Stay tuned for those to drop. And until then, cheers and good science. Speaking of Mol Bio is produced by Matt Ferris, Sarah Briganti, and Matthew Stock.