Webinar 02 Transcript (English)

Comprehensive Coverage of Exosome Purification and Exosomal RNA Isolation from Different Types of Liquid Biopsies

Presented byBastien Paré with Dr. Moemen Abdalla

Tom Hunter: INTRODUCTION

Hello everyone! Welcome to the second installment of Norgen Biotek's new webinar series.

My name is Tom Hunter and I'm the International Sales and Marketing Manager here at Norgen. We are delighted to have you all joining us today. Thanks for your time.

Today's presenter is Bastien Paré who joined our R&D team this last year and is working extensively with liquid biopsies, exosomes and RNA purifications, small RNA NGS and much more here at Norgen Biotek.

The title of today's presentation is: "Comprehensive Coverage of Exosome Purification and Exosomal RNA Isolation from Different Types of Liquid Biopsies". We hope you find the presentation today interesting and informative.

Don't forget that at the end of today's presentation we will hold a question and answer session with Bastien and Dr. Moemen Abdalla, as well, so please add your questions anytime during the presentation and we will be delighted to assist you during the Q & A.

Finally, we will offer a recorded version of this webinar and a link will be provided afterwards.

So, please welcome Bastien who will take it from here. Thanks, Bastien.

Bastien Paré:

Slide 1: Opening Title slide
(1:12)

Thank you, Tom, for the presentation.

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Sorry, there was a little hiccup here, so I'm going to start again from the beginning, so thank you, Tom, for the presentation. As he said, the webinar of today will be "Comprehensive coverage of exosome purification and exosomal RNA isolation from different types of biopsies".

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When we were talking about liquid biopsies or bodily fluids, it is important to know what kind of molecule is in circulation and what kind of molecule these bodily fluids and liquid biopsies are containing.

A part of these molecules are cell-free RNA and cell-free DNA that are shed from cells either after program death, so after apoptosis, or from necrotic cells after necrosis, which is usually associated with a pathologic state.

Regarding the cell-free DNA, the DNA is usually bound to histones, and that will allow the DNA to be stable in circulation. Regarding the RNA, the cell-free RNA, to remain stable, it's going to be, usually, bound to protein as the Argonaute protein or AGO.

RNA can also be contained in extracellular vesicles as exosomes which are secreted by living cells, by cells. Exosomes are known to contain miRNAs, proteins, as well as mRNAs.

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Here, we present some key features of extracellular vesicle populations.

Today, we're going to talk mostly about exosomes, but it's important to know that from the extracellular vesicle population, there are also microvesicles, as well as apoptotic bodies.

There are some markers associated with exosomes as CD9, CD63, CD81, Alix, as well as TSG101.

Exosomes are extracellular vesicles with a diameter of 40 to 100 nanometers. They are multivesicular bodies which are known to contain proteins, RNA, and miRNAs. To detect them, usually, we are going to use either FACS, electron microscopy, Western blot for specific exosomes, enriched markers, or NTA. And, they are released by exocytosis.

Regarding microvesicles, they have a diameter of 100 to 1000 nanometers. Their markers are usually Annexin V, some integrins, some selectins, as well as Flotillin-2. They are membrane blebbing, so they will be round in shape. They do contain protein, RNA, as well as miRNAs. And, they can be detected by FACS and electron microscopy. ANd, usually, their mechanism of releases, they are going to be budding from the plasma membrane.

And, finally, the last population of extracellular vesicles are the apoptotic bodies. They have a diameter of 1000 to 5000 nanometers. The markers associated with apoptotic bodies are Annexin, some DNA, as well as histones. Regarding their shape, they are going to be shink cells because apoptotic bodies are dead cells. They contain cell organelles, proteins, DNa, RNA, as well as miRNA. We usually detect them using FACS and electron microscopy. And, the mechanism of release, of course, apoptotic bodies are dead cells, so they are going to come from cell shrinkage and also from plasma membrane blebbing during cell death.

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But, if we go and we check further on exosomes, what are they, in fact?

Exosomes are small membranous vesicles secreted by most cell types, including neurons. They can be isolated from cell culture media and bodily fluids such as urine, plasma, saliva, as well as cerebrospinal fluid or CSF.

Exosomes contain an array of different proteins: some are specific to the cell type of origin - the cell type that secretes the exosomes - while others are common across all exosomes.

They also contain heat shock proteins, adhesion molecules, metabolic enzymes, cytoskeletal proteins, and are heavily enriched in tetraspanins such as CD9, CD63, and CD81, which are considered by some people as exosomal markers.

In addition to their protein content, these vesicles have recently been shown to contain mRNAs, as well as miRNAs.

An exosomes can be isolated from circulating fluids such as serum, urine, and cerebrospinal fluid, they provide a potential source of biomarkers for different pathologies.

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And, these extracellular vesicles, if you want to study them, how are you going to be able to purify them? Well, there are some methods and protocols already available for exosomes or extracellular vesicles purification.

The actual gold standard for exosomes purification and the most studied purification method is ultracentrifugation. It's a time-consuming method which will lead to low throughput and some contamination from proteins or cell-free DNA or RNA, but it's still the gold standard right now.

Another purification method is the use of polymers as the ExoQuick reagent or kit sold by Invitrogen. They do allow for a high throughput at an acceptable price, but they will lead to co-precipitation of polymers and the co-precipitation of proteins.

You can also use filtration columns to purify exosomes or extracellular vesicles. Filtration columns are quick, they will lead to a high throughput, and they can allow for size selection, but they are very expensive and, of course, if you do selection of size, well, the exosomes or the extracellular vesicle population you have will be size - dependent on the size.

And, you can use antibodies to purification exosomes. Antibodies allow for a quick purification, it's highly specific, but the price is very high, so there's a high cost to use exosomes - exosomes, sorry - antibodies, and of course, you're going to be able - you will need to purify a subset of extracellular vesicles because you need to keep in mind when you are purifying exosomes or extracellular vesicles, using antibodies, you are going to purify them based on the presence of a specific protein or some specific proteins under the surface or membrane, but if - some exosomes don't express - don't have these proteins under the surface, they won't be purified, so there's that to keep in mind.

And, finally, the last purification method, is ultrafiltration which will lead to high purify and which will lead, also, to high homogeneity, but it's going to - you're going to get a low throughput and a loss or very low diversity.

So, all in all, given all the attention drawn to the field, there is still no universally accepted standard in isolating exosomes derived from different liquid biopsies, and that's where Norgen Biotek kits come in.

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Norgen Biotek offers a lot of kits to purify exosomes from different liquid biopsies being plasma or serum, urine, cell culture media, or saliva.

Norgen Biotek technology is based on the isoelectric point of exosomal proteins, which is different from the isoelectric points of histones or other proteins. Norgen kits are based on the proprietary silicon carbide technology and offers a simple and fast verification of exosomes without the use of either ultracentrifugation, filtration, antibodies, or long incubation.

The purification exosomes for multiple applications such as Western blots, electron microscopy, NT analysis, as well as protein, and RNA isolation.

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And, Norgen Biotek kits offer simple and rapid exosomal RNA purification. So, let's say you have a liquid biopsy being either plasma, serum, or urine. Using Norgen Biotek kits to purify exosomes, you're going to collect purified exosomes without purifying, at the same time, the free-circulating RNA, which is important because when you analyse exosomal RNA, you don't want to have the free-circulating RNA. You want to analyse only what's inside the exosome. So, that's something to keep in mind. And, Norgen Biotek kits, they do offer exosome purification kit that will allow for the purification of only exosomes and not free-circulating RNA. And, we do also offer RNA purification kit which will lead to a very good extraction of exosomal RNA.

What's to keep in mind about Norgen Biotek kits is that the purified exosomes that you're going to get are without contamination, linear, and scalable.

As you can see at the bottom left, with the NT analysis, either you are using 1mL of plasma, or 10 mL of plasma to purify exosomes. You won't get more contamination. The kit is scalable, but you're going to get more exosomes - highly pure exosomes - which are going to be eluted in a very simple buffer that is compatible for downstream applications including microscopy.

And, after NGS analysis, you will see that the exosomal RNA that you are going to collect is without any contamination and without any inhibitors which will allow for NGS analysis. We will come to that a bit later.

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Here we are presenting the results that were published by Royo and collaborators in 2016, where they collected urine exosomes and they did purify exosomal RNA from urine using Norgen Biotek urine exosome RNA purification kits. And, their conclusion was that either when you are analysing protein by Western blot or genes using PT-qPCR and enrichment of exosomes using Norgen's technology resulted in a better signal for protein and RNA when compared to other isolation methods as ultracentrifugation, lectin-based methods or protocols, or other commercial products.

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And, finally, Norgen Biotek [exosome purification kits] do allow for highly pure exosomes obtained in less than an hour and presenting with a very good size distribution, usually ranging from 40 to 100 nanometers.

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Norgen Biotek also offers a solution for the study of exosome purified from cell culture media. As some of you may know, FBS - so, [fetal bovine serum] used in cell culture media - must be depleted from bovine exosomes in order to only purify human-derived exosomes to minimize the interference with downstream analysis and downstream experiments.

So, here at Norgen, we have developed an FBS exosome depletion kit which will allow for exosomes depletion from FBS at a lower cost than exosome-depleted FBS that you can buy and is commercially available.

Here as an example, you can see on the left, Hela cells cultured in a regular cell culture media with standard FBS and on the right you can see the same cells cultured in a regular cell culture media with FBS that has been depleted of exosomes. And,what's interesting to see is that you can see there is similar growth and identical cellular morphology either if you're using deleted-FBS, exosome-depleted FBS, or standard FBS, which means that the exosome depletion kit from Norgen Biotek won't deplete the serum from any nutrients that the serum will contain for the cells.

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And, we can also say Our technology is also very efficient, as shown here, where most exosomes associated miRNA that we analysed, being miR26a, miR30a, miR92a, 23a, as well as 122 are not detectable by RT-qPCR, when compared to a depleted FBS sold by one of our competitors. So, exosome-depleted FBS with Norgen's FBS exosome depletion kits, they do have undetectable bovine miRNA.

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And, of course, there is something very important to keep in mind and take into consideration when you're working with exosomes. In fact, there are four points that I'm going to go through in this presentation.

First, you need to take into consideration the exosomes purification method you're going to use when working with exosomes. You want this purification method to be strong and reliable without purifying cell-free RNA.

You need to keep in mind that the exosomal RNA purification method will be very important. Since exosomes do contain a very low concentration of RNA, so you will want a kit that will be efficient to purify very low concentrations of RNA.

You might want to quantify this RNA which is present on a very low concentration, so there is some consideration to take into account when you quantify RNA derived from exosomes, as well as when you try to assess the quality of the isolated RNA.

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So, here are some RNA purification technology considerations for exosomes analysis.

First, exosomes, or extracellular vesicles, are sub-populations of liquid biopsies that are presenting very low concentrations of RNA.

There is still no universally accepted view on what is the RNA content inside exosomes, or extracellular vesicles. We know it's fragmented RNA, but is that RNA large, is that RNA very short, or is it miRNA, and is there a presence or absence of ribosomal RNA.

These are things that are still under research by many research teams, but it is important to keep in mind that when you want to purification exosomal RNA, the technology you're using must be very sensitive and allow for size diversity.

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Which allows me to talk about the most common purification kits, being the phenol:chloroform technique or the silica column chromatography.

Most bodily fluids RNA extraction products are based on these technologies that have been developed more than 30 years ago, that were not made for liquid biopsies and RNA extraction.

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One example of that is the conclusion that Kim and collaborators had in their paper published in 2012 in *Molecular Cell*, where they showed that they used phenol:chloroform for RNA extraction shows an important bias leading to loss of RNA diversity.

In fact, they showed that low GC content RNA are selectively lost during extraction. And, their conclusion was that phenol:chloroform extraction procedures tend to show a bias in RNA recovery based on GC content, which includes, of course, miRNAs, which are, from what we know, the main RNA present in exosomes.

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Regarding the use of a silica column for sample preparation, the silica columns, they do show a size bias. There's a loss of a lot of small RNAs, unfortunately.

Either you're analysing your RNA using a regular agarose gel or on a bioanalyzer. When you compare total RNA to RNA purified on a silica column, you can easily see at the bottom that small RNAs, which including siRNAs and miRNAs are not detected or are missing from the samples when you purify RNA using silica columns.

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And, the phenol:chloroform technique or the silica columns for sample preparation are not designed for body fluids isolation or exosomal RNA application. And, most of the kits that are available on the market right now, they are using these two technologies. Most of them are going to use phenol.

When they use a column chromatography, they are going to use silica, and most of them, if you want to achieve sensitivity in your RNA extraction from samples having a very low concentration of RNA, they are going to use carrier RNA, too.

Most RNA sample preparation products, unfortunately, they adapted to the latest generation technologies to allow for Next Generation applications, but are not made for it.

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They are not efficient, and that's where the silicon carbide technology developed by Norgen Biotek comes in as a matrix for nucleic acid and protein purification for Next Generation applications.

The use of silicon carbide will allow for a very good diversity. The silicon carbide can bind all types of DNA and/or RNA, including short RNA as miRNAs, without using phenol.

The silicon carbide shows very good sensitivity, so you can extract RNA from exosomes and even from single cells without the use of any carrier. And, the silicon carbide can be used for different applications as spin columns, 96-Well plates, and slurry, which is mainly used for liquid biopsies and body fluids.

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Based on the results published by Kim and collaborators in 2012, that led us to do a small experiment here in the lab where we compared different kits of RNA extraction kits from Norgen Biotek, Qiagen, and Ambion after spiking samples with synthetic RNA presenting different GC content.

As you can see here, Norgen's silicon carbide technology allows for a better diversity of miRNAs, especially miRNAs with low GC content. Norgen's biotechnology doesn't show any bias to bind different GC contents of RNA. The proprietary technology has been developed in order to allow for the purification of both large and small RNAs, which is not the case for all the kits available on the market right now.

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And, when you compare the silicon carbide technology on a column to the silica column, the regular one, you can see either on an agarose gel or after a bioanalyzer analysis, that Norgen Biotek's silicon carbide allows for the purification of small RNAs including miRNAs and short and differing RNAs, which is not the case for silica-based columns.

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We developed our kits keeping in mind that we need a flexible entry of workflow to customize individual needs. So, either you are going to try to purify exosomes and exosomal RNAs from urine, plasma or serum, or cell culture media, Norgen Biotek will offer an exosomes purification kit that will allow for the purification of very, very pure, without any contamination, exosomes which can be used, then, to purify RNA using Norgen's best-in-class purification and that RNA, here at Norgen Biotek, we do have a workflow for small RNA sequencing.

But, we kept in mind that not everyone will use Norgen Biotek's exosome kits to purify exosomes, and during the development of our RNA extraction products, we took that into consideration.

So, our best-in-class purification kit can be used for RNA extraction of exosomes that have been purified using other purification techniques such as ultracentrifugation, antibodies, as well as filtration.

Norgen Biotek RNA purification technology has been adapted for all the available exosomes purification methods allowing for optimal recovery of total RNA, including, of course, small and miRNAs, which can be used for small RNA sequencing.

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Here, in order to show what we do - we pretended we extracted or purified extracellular vesicles using ultracentrifugation and we purified RNA using either Norgen Biotek purification kit using the silicon carbide technology, either a kit using the phenol + silica techniques, or just the phenol. And, we did small RNA sequencing using our workflow. And, what's interesting to see, in fact, is that using Norgen Biotek's silicon carbide technology for RNA purification, there's a unique number of miRNAs that were purified, which means a higher chance of discovery of either novel miRNAs or biomarkers in research.

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And, we also did exosomal miRNA transcriptome analysis in order to compare different exosome purification technologies.

So, we did - compared Norgen exosome technology to the ExoQuick from System Biosciences, a Qiagen kit, as well as the Life Technologies kit. And, what's important to note here, is that there is a significant overlap of miRNAs and each method has its own set of unique transcripts.

So, using Norgen Biotek kit, we were able to detect 18 unique miRNAs. Using ExoQuick, we were able to detect 10. And, using Life Technologies, we were able to detect 1, and using the Qiagen kit, we were able to detect 35.

So, of course, 35 is higher than 18. But, again, we did that comparison in order to compare the different exosomes purification technologies and to insert a question: which is better - do you want to detect more miRNAs or less miRNAs, because, of course, you want to keep in mind, always keep in mind that depending on your exosomes purification technique, you might purify at the same time cell-free RNA, which is not exosomal RNA, which lead to a higher count of unique RNAs detected, but these RNA may not be specifically from exosomes.

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And, when we do a breakdown of the figure before, we can easily see that there is a significant overlap of detected miRNAs when we compared Norgen Biotek exosome purification kits with other kits.

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To compare the miRNA profile of exosomes isolated by different methods using small RNA-sequencing and to compare

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We also use plasma-derived exosomes in order to do miRNA profiling using the Illumina Next Generation Sequencing platform.

The object of our small study was to compare the miRNA profile of exosomes isolated by different methods using small RNA sequencing, as well as to compare the efficiency of different RNA purification methods for miRNAs recovery from an exosome sample.

So, we used five methods of exosome enrichment from plasma: ultracentrifugation; Qiagen technology, which is a size filtration column; System Biosciences ExoQuick, which is a reagent; Norgen Biotek’s silicon carbide technology; and an antibodies-based technology.

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So, we purified exosomes from plasma. Plasma was used at equal volumes for exosome enrichment by different methods listed before. RNA was isolated in duplicates from the enriched exosomes using two different kits - either a Qiagen kit, which is a combination of phenol extraction and silica column purification, which takes 40 minutes to an hour to do, and Norgen's silicon carbide column which requires no phenol. And, that takes between 15 and 20 minutes for the isolation.

We did the library preparation using Norgen's Small RNA Library Prep kit for Illumina. The sequencing platform we used, is the sequencing platform - that we have here in-house at Norgen Biotek and for which we offer services. And, we used an Illumina NextSeq 500. And, the data analysis pipeline was the exceRpt small RNA-seq Pipeline.

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And, when we compare miRNA diversity based on different exosome purification methods, there's a lot of things we need to take out of that.

First thing first, when we purify RNA, as shown on the left, Norgen's patented resin technology recovers small RNAs from exosomes efficiently. In fact, using Norgen's silicon carbide technologies when compared to phenol + silica based technologies, the silicon carbide allows for the purification of a higher number of unique miRNAs.

And, when we compare the different purification methods, each exosome purification method or protocol shows a different panel of miRNAs, but what's important to keep in mind is every exosome purification method won't be perfect, and some of them can lead also to the purification of cell-free RNA, which, again, is not something we want when we're analysing or purifying exosomal RNA since we want the RNA present only within the exosomes.

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And, when we compare the different methods, the different exosome purification methods, two things to take away from that, exosomes - they do contain a significant amount of miRNAs, and each exosome purification method yields a different set of exosomes or exosomal RNA, which is shown here....

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...by that heat map, where we did exosomal miRNAs profiling using Next Generation Sequencing. And, what's interesting to see on the left, you can see one specific profile that is very, very different than the profile on the right.

The profile on the left is associated with surface protein-based separation of exosomes as the antibodies technique - or protocol or the Norgen Biotek silicon carbide technology.

On the right, you have a completely different profile of miRNAs. While this profile is associated with exosome purification or separation based on vesicle size, using either ultracentrifugation, System Biosciences ExoQuick, or Qiagen's exoEasy.

So, liquid biopsies of ultra-low RNA content such as exosomes can be used for small RNA expression profiling using Illumina Next Generation Sequencing technology, and as shown before with the heat map, there is a "signature" of small RNAs, as well as, of course, miRNAs, based on the exosomes isolation method.

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In summary:

Illumina Next Generation Sequencing platform is fueling the rapid growth of personalized and precision medicine, as well as revolutionizing the use of molecular biology approaches in order to tackle problems in fields such as agriculture, diagnostics, and public health.

To fully unlock the true potential of Next Generation Sequencing, the isolation of high quality RNA from either low nucleic acid concentration samples or difficult to extract samples is a must. Norgen Biotek has been supplying best-in-class RNA products that are optimized for NGS applications.

The combination of Norgen's sensitive RNA purification platform with Illumina's NGS platform has enabled robust RNA/small RNA profiling from liquid biopsies for biomarker discoveries.

But, again, when you want to do Next Generation Sequencing - so now we've been through the exosomes purification techniques, we've been through the RNA purification techniques, and as mentioned earlier, before doing NGS or before doing any PCR or RT-qPCR or whatever analysis you want to do with your RNA, you need to be able to quantify that RNA and you have to be able to check the quality of your RNA.

And, how do you check - how do you quantify exosomal RNA, which is known to be present in very, very low concentrations from 1 to 100 picograms per microlitre.

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Well, of course, we know quantification of nucleic acids from liquid biopsies is challenging.

And, unfortunately, most of the technologies on the market right now won't allow for the quantification or the proper quantification of exosomal RNA. Using regular spectrophotometry, you're going to be able only to quantify nucleic acids from 20-50 nanograms. Nanodrop goes down from 1 to 10. The ribo or pico Green will be able to quantify down to 1 nanogram. And, the Bioanalyzer down to 50 picogram.

But, what if your exosomal sample, they do have one, two or three picogram per microlitre, how do you quantify that?

Well, Norgen Biotek developed a PCR - qPCR based quantification method available for both RNA and DNA quantification. That allows for quantification of RNA, or DNA, of course, but in that case RNA, down to one picogram and even down to 100 fantogram, which will allow for a very strong and specific quantification of exosomal-derived RNA.

Unfortunately, using that technique, it's a bit hard to see if you have a good quality of your RNA, but....

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...here at Norgen Biotek, we've also developed an application note that explains how to determine the quality of your nucleic acid using qPCR or RT-qPCR.

And, the way to do so, in fact, is to allow for a higher input of RNA - higher concentration of RNA in your PCR reaction mix. So, technically when you add more RNA, your CT value should go down, which will lead to the conclusion that you have RNA of very good quality, not degraded and without any inhibitors.

But, if you add more RNA into your PCR reaction and the CT value goes higher or goes up, then that means there is elute - in your RNA elute the presence of inhibitors. So, that's using PCR or RT-qPCR, you can easily determine the quality of your purified nucleic acid, even if you have very low concentrations of RNA in your samples. And, you might ask why I'm not using the ratio 260:280 from the nanodrop, well, of course that's going to give you a number, but it might tell you that your RNA is not of good quality, although it would be of good quality, and without inhibitors.

The reason why is because the nanodrop is also using the RNA concentration in order to calculate for the ratio and to make sure the RNA is of good quality.

The Bioanalyzer also offers a way to check for the quality of your RNA, which is the RIN value, but the RIN value is based on the 28s and the 18s RNAs, which are not, unfortunately, not present in exosomes, so the RIN value will be very low for exosomal RNA, although it might be of very good quality and without inhibitors.

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And, finally, I just want to give you a quick overview of what we offer here at Norgen Biotek as a Next Generation Sequencing platform for small RNAs and miRNAs, because we do offer that service.

Usually, how we like to work with our customers for Next Generation Sequencing is that we start with an initial consultation where we give expert advice on sample handling, processing,and experimental design for the best data outcome is given.

Then, we proceed to the RNA isolation/RNA submission. You can either submit isolation RNA or send a sample for RNA isolation, and our team will guide you through what is required different sample types.

We then, in-house, quantify the RNA and do quality control on it. So, it's going to be quantified and assessed for quality.

Then, we do library prep sequencing using our Illumina platform. Data analysis which will include, to say the least, filtering, aligning and mapping, annotating with statistical analysis, and a final report - a final comprehensive report detailing the analysis and results can be sent to the customer after sequencing.

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So, that ends the webinar for today. Thank you very much for joining us.

For more information, you can go on our website at norgenbiotek.com. You can always contact us info@norgenbiotek.com, orders@norgenbiotek.com, or techsupport@norgenbiotek.com. You can always call.

And, using the promo code of WB0219, you can receive up to 15% off your first order.

Thank you for being there.