Cell and Molecular Biology
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Week 09
Central Dogma of Molecular Biology (Part 1)
Lecture - 33
Structure of RNA

Hello everyone, this is Dr. Vishal Trivedi from the Department of Biosciences and Bioengineering at IIT Guwahati. In today's lecture, we are going to discuss the properties of RNA, the structure of RNA, how you can isolate RNA from the cell, how you can characterize RNA, and how you can estimate RNA, and so on. So, why is it important for us to understand RNA? Because RNA is mainly responsible for the synthesis of proteins. So, if you want to do an experiment related to expression studies and other kinds of studies, then you are supposed to study the RNA. Now, when we talk about RNA, we are actually going to talk about the three different types of RNA.

So we have three different types of RNA. We have transfer RNA, commonly known as tRNA. So, this is called transfer RNA. Then we also have ribosomal RNA.

Or it is called rRNA, and we also have messenger RNA, or it is called mRNA. The mRNA is actually going to provide the message, or it is going to provide the information on which sequence we are going to add the amino acids. So it's actually going to provide information about the synthesis. which means which amino acid I should add, like that kind of information. So, for example, if I want to start writing a letter, I have to first know what the sentence is.

Right. So if I know the sentences, then my brain is actually going to read those sentences. And that sentence is nothing but this message. Right. And then I am going to bring ABCD like that.

OK, so that ABCD information is correct. Now you are actually going to read about the transfer RNA and the ribosomal RNA. So transfer RNA is actually going to bring the amino acids in the same sequence as the one given here, right? So, if it says you bring the alanine, it is actually going to bring the alanine. If it is going to say the methionine, then it is actually going to bring in the methionine. So, this is actually going to bring the amino acids, and then these amino acids are actually going to be joined by the ribosomal RNA, right? And you know their amino acids are actually going to join by a bond that is called a peptide bond.

So basically, the job of the ribosomal RNA is to form the peptide bond between A and B,

and that is how it is actually going to start synthesizing the protein molecules. So these are some basic points or a brief overview of the function of these RNA species. One, it is actually going to provide the message. Information on synthesis, such as the sequence in which I should add the amino acids, and which amino acid you are going to provide, will be included. The transfer RNA is actually going to bring that particular RNA, and the ribosomal RNA is going to collect the information from the transfer RNA and messenger RNA.

That is how it is going to join. The amino acids, with the help of a peptide bond, form the sequence, and that is how it actually contains the information, okay. So, this is just an alphabet; actually, there is no amino acid with the letter B, right? And so on. Today, in today's lecture, we are not going to cover the structure of transfer RNA or ribosomal RNA. We will only focus on the messenger RNA.

We will take up the structure of transfer RNA and ribosomal RNA when we actually discuss the translation because all three RNA species actively participate in the translation process. So we are only going to focus today in today's lecture; we are only going to focus on the messenger RNA. In our subsequent lecture, we will focus on transfer RNA and ribosomal RNA when we discuss translation. So, we'll talk about the structure of the messenger RNA. So messenger RNA is actually, as I said, it should have the information for the synthesis, right? It is going to provide information about the synthesis of proteins, which it is actually going to take from the DNA, right? So, that information is originally present in DNA, but that information will then be taken up by the messenger RNA.

So, messenger RNA has three distinct parts. This is the 5' prime end and this is actually the 3' prime end, okay. And remember that we discussed in detail the RNA structures or the composition of the RNA when we were talking about the DNA, right? So RNA is actually also a polynucleotide molecule. RNA is single-stranded in the majority of cases, and RNA also has phosphodiester linkages and a phosphate backbone. So RNA is also going to be made up of the sugar, phosphate, and base, right? As far as the base is concerned, the RNA is actually going to have A, G, U, and C.

It does not have the T, so there is no T present. Okay, so T is absent in the case of RNA, whereas the T is present in DNA. Instead of T, it actually has uracil. Okay, and that is the basic difference between RNA and DNA. Other than that, it is going to be a single standard.

So there will be extensive secondary structures present in the RNA species. And as far as the structure is concerned, the RNA is going to have a five-prime cap. So this is the cap

that is actually going to protect the RNA sequences because RNAs are very, very susceptible to RNA molecules. And then it is actually going to have the 5' prime UTR. So the 5' prime UTR is a place that is actually going to provide the docking site for RNA polymerase.

Detail how the RNA polymerase is going to sit and how it is going to recognize the promoter regions, and all that we are going to discuss when we talk about translation. So, in the 5' UTR, you are going to have the promoter, correct? And there is a definite composition of the promoter. So it's going to have the data box. It's going to have the minus 10 region, minus 35 region, and so on. So all that I think can be discussed when we are discussing the transcription and translation.

So a promoter can be a strong promoter or it can be a weak promoter. So, promoters are actually going to provide a docking site. For the translation's initiation site. So it's going to provide the translation initiation site, and it's going to allow the binding of the RNA polymerase. So it's going to provide the docking site for protein synthesis.

So it's going to provide a docking site for the ribosomal machinery. And this was going to be a promoter, so it is actually going to be a strong promoter or a weak promoter, and it is actually going to provide the docking site for the translational machinery. Next to this, you are actually going to have the coding sequence, so this is a region that is going to be a coding sequence; this is the region that is going to give you the protein, so it's actually going to provide the information in terms of the genetic code. and these codes are actually going to be read by the ribosomal machinery and as well as by the tRNA. And that's how it is actually going to help you in the synthesis of protein.

So genetic information is encoded in the form of genetic code, and each genetic code actually corresponds to the amino acids. These amino acids are then going to be added into the ribosomal machinery with the help of the peptide bond, and that is how it is actually going to be synthesized. And then you also have the three prime UTRs. So three prime UTRs are actually going to be the regulatory site, which is actually going to provide the regulation of this whole translation process. And then at the three prime end, you are actually going to have the polyadenylation site.

So polyadenylation is very important because, with polyadenylation, you can actually have the addition of the ACE starting from 50 to 200. And depending on the polyadenylation, you are actually going to decide the age of the messenger RNA. Because this is actually going to be chewed, remember that it is from this side by an RNA. So if the RNA is processing this messenger RNA because our messenger RNA is going to be present in the cytosol outside the nucleus. So RNA is actually synthesized by

a process of transcription inside the nucleus.

Then it is actually going to be transported outside into the cytosol, and then it is actually going to provide a docking site for the protein synthesis machinery, which means it is actually going to allow the assembly of the ribosomal machinery, like the small subunit and the large subunit. And that will happen on the 5 prime UTR. And then it is actually going to synthesize the proteins, but how long this messenger RNA is going to remain active in the cytosol will be decided by the 3 prime poly A tail, okay. So, this region is called the poly A tail, okay. And this polyethylene is going to be, or the ribose RNAs, which are very active within the cytosol, are actually going to chew these RNAs from the 3 prime end.

The moment they are actually going to be chewing up like this, once they hit the coding sequence, then they are actually going to start; this messenger RNA will not be useful for the synthesis of the protein. Because now it is actually going to start synthesizing the, uh, you know, the cryptid protein or the truncated proteins, and that may not be good for the cell. So the length of this polyadenylation or the length of the poly tail will actually decide the age of this particular messenger RNA, or I would say the stability of this messenger RNA within the cytosol. So there are messenger RNAs where you have a very large number of ACE, and they will actually remain in the cytosol for a very long time so that they will keep expressing the protein. So some of these messenger RNAs belong to the housekeeping genes.

For example, you have the messenger RNA for actin, myosin, and LDH. So these are the proteins that are required, and there is a huge demand for these proteins, and that's why they are supposed to be synthesized. And since they are housekeeping genes or actually housekeeping proteins, their level is going to determine the health of that particular cell. And that's why they are actually going to have a huge number of polyethane or a huge number of amino acids, A-residues in the polyethane. Now, polyethylene is a very, very interesting tool because it also provides stability to messenger RNA.

At the other end, it can also be a tool to purify the messenger RNA from the cytosol. So that we can do it with the help of an affinity column. And that is what we are going to discuss now. So how are we going to purify the messenger RNA from the cytosol? So there are two methods by which we can isolate the messenger RNA. One, we can actually use the affinity column.

And we can use the affinity DT columns. And the other approach is that we can actually use the trizone method. And this linker has the T residues, which means it actually has thymine. So when you have thymine, you know that A always pairs with T and G always

pairs with C. Utilizing this information, if you have the A's present on the messenger RNA.

So what you can do is actually have the beads and put the beads into the cytosol. So what will happen is that you are actually going to have the binding of A's, which are a part of the polyadenylation tail, and it is actually going to have the messenger RNA. So, this is the messenger RNA, and it is going to have this right, and ultimately what you are going to do is you will actually be able to do the elution, and at the end, what you are going to have is this. So, this is actually going to give you the complete pool of messenger RNA. This is another method called the trizole method, where you are actually going to use the trizole, and that is how you are going to isolate the messenger RNA.

So, in both of these methods, we are going to discuss how you can isolate the messenger RNA from the cytosol. First method, so this is method one, right? And this is method two. So for the first method, what you're going to do is test grow the cells. So these are the target cells.

You can also have the tissue. So depending on what kind of material you are using. So if it is a tissue, then you are actually going to grind the tissue so that it will give you the single cell suspension. Sometimes you might have to use the enzymes and the kinds of treatment that we are not discussing here. So, if it is starting with the tissue, for example, you started with the liver, right? So, if it is started with the liver, then it has to be ground fine with the cell mortar or the homogenizers. And then the liver is actually going to provide you with the single-cell suspension.

And then, from the single suspension, you are actually going to use it in the same way as you are going to use the cell from the cell culture. So you're going to put them into a lysis buffer. Mostly, the lysis buffer contains SDS and also contains the protein scale. And it also contains the SumtimeHDS or SumtimeTitronX100.

Okay. So, it is basically going to contain the detergent, the proteinase K, and it is also going to have the binding buffers. So, you lyse the cells with the lysis and binding buffer, and then you are going to—so this is what we have shown here, right? If you have a tissue, you can just do the homogenization so that you will have a single cell suspension, and then you can incubate it. Once you incubate this, it is actually going to lyse the cells, and you are going to have the cell lysate. Okay, so in the cell, I said you can do the spinning at, for example, 1000 rpm, so that is actually going to remove the nucleus, and then it is actually going to give you the cell lysate. Because the nucleus is useless and is actually going to increase the contamination, if you remove the nucleus, you are actually going to get rid of the DNA, right? And then you take the messenger RNA, put it into the

binding buffer, and then you are actually going to have the oligodT beads.

And I just explained that the oligodt beads are going to have the agarose beads and that it's also going to have the linker. That linker is actually going to have the T-residues attached to it, okay? This means that these linkers are actually going to have very strong and specific binding for the A-residues, okay. So, when you do that, the messenger RNA that is present in this particular cytosol is not specific for a particular messenger RNA. It's actually responsible for all the messenger armies. And that's how it is actually going to bind the messenger army.

So, this is the messenger army. So they will interact with one another. And then you are going to do a wash with the buffer because there could be some non-specific interactions where you can do a wash with the buffer. That washing can be done with the buffer and salt. So you can actually add some salt to reduce the non-specific interactions.

And then you are actually going to do the illusions. OK, so once you have the pure sample, you are actually going to create the illusion. So illusion can be done at this step. You collect the beads, discard the supernatant, and then perform the illusion. So, for example, you can add the quality, or you can actually add the thymine.

And then you can suspend that in the illusion buffer. And the elution buffer is going to allow, or it's actually going to break, the hydrogen bonding between the poly T tail, which is attached to the beads, and the poly A tail, which is present on the messenger RNA. And that's how the messenger RNA will be eluted, and then you can actually take this pure messenger RNA for further downstream applications like RT-PCR, and you can use that for other kinds of applications. So this is exactly what people were doing when you actually asked them to do the COVID testing. So they were taking your saliva and other kinds of samples, and then they were doing this process to isolate the messenger RNA, and then they were doing the RT-PCR with the help of the primers for COVID. And that's how they were saying that if they were getting the amplifications of the cDNA, then they were actually saying that it was COVID-positive.

Anyway, that is a separate part that we are going to discuss when we talk about real-time PCR and reverse transcriptase. RT-PCR and we will also discuss how you can use RT-PCR for this kind of application. So this is the first method where you are actually going to use the affinity column to purify the messenger RNA from the cell lysate or tissue. Now let's move on to the next step; the next method is called the TRISOL method. The RNA isolation by the trisol method uses trisol, which is also called tri-reagent, for the isolation of total RNA.

Trisol is a mixture of gonidine thiocyanate. And phenol, which effectively dissolves the DNA, RNA, and the protein upon homogenization or lysis of the tissue samples. After adding the chloroform and centrifugation, the mixture separates into three phases, with the upper clear aqueous phase containing the RNA, the interface containing the cell debris, and the lower organic phase having the protein and the liquids. The next step in the extractions is the washes and the precipitation of the RNA. The first part of the protocol from the homogenized tissue in triazole to the point of an RNA pellet in 75% ethanol takes less than an hour. The RNA can then be stored for a long period of time at minus 20 degrees Celsius.

So RNA is very stable when you are isolating it with the trisol method and putting it into the 75 percent ethanol. The same protocol can be used for RNA extraction from cell culture. So if you want to remove the DNA, you can actually treat the sample with DNA. And that's how it's actually going to remove the DNA portion. So this is what it's actually going to say: that if you have the grinded adipose tissues, for example, this is a tissue, right? So in step one, you are actually going to add the reagent, right, and vortex, then wait five minutes at room temperature.

And then it is actually going to give you the aqueous phases, and you're going to have the different types of buffers. And what you're going to see here is that when you have the phases, you're going to have two phases: one is the RNA phase, and the other one is the chloroform phase. And in this phase, you are actually going to have the protein plus lipid, whereas in the aqueous phase, you are going to have the messenger RNA that you can transfer; this aqueous phase then allows you to use that by precipitation with the 75% ethanol. You can actually air dry this pellet and then dissolve it in an RNase-free buffer, and that's how you'll obtain the RNA. So let's see what the different methods or protocols are, right? So this procedure is very effective for isolating the RNA molecules of all types from 0.

1 to 15 KB in length. However, there are commercial kits that enable simple RNA extractions using a column that binds the RNA and so on. That is anyway we have discussed, right? So, what are the requirements? So, the first thing is that you actually require the triazole to obtain the 1.5 ml appendox, you require the centrifuge to obtain the chloroform, isopropanol, RNA-free water, micropipettes, and the tips, as well as the test specimen. So, you require either the tissue or the cell. So the first step is that you are going to either take the tissue or the cell culture cells, and then you're going to do the homogenization or the lysis.

And once you do the vertexing and all other kinds of things, then you are actually going to add the trisol, and that is actually going to have the phase separations. So you're going

to have the aqueous phase, and then you're also going to have the organic phase. In the organic phase, you're going to have the lipid and the protein, right? And then you collect the aqueous phase, add the ethanol, and that's how it's going to form the RNA pellet, which you air dry, and then you add the RNase-free water to resolve things. Procedures - In step one, you are going to add the triangle reagent to the cell and incubate it at room temperature for five minutes.

Then, you transfer the cell lysate to a 1.5 ml centrifuge tube and add 0.2 ml of chloroform. so this is what you're going to do right in the step one you're going to add the chloroform mix it thoroughly and incubate at room temperature for five minutes then you can diffuse the mixture to the centrifugation at 12,000 g for 15 minutes at 4 degrees Celsius, transfer the aqueous phase containing the total RNA to a fresh tube and precipitate the RNA by adding 0.5 ml of isopropanol, followed by incubation at room temperature for 10 minutes. Then you centrifuge the precipitate at 12,000 g for 10 minutes at 4 degrees Celsius.

And then you discard the supernatant, air dry the RNA pellet for 10 minutes, and resuspend it in 20 microliters of RNA-free water. Remember that this is very important. You can either purchase the RNA-free water from commercial vendors or prepare the RNA-free water in a laboratory. So it's not a very difficult part. Then you perform the agarose gel electrophoresis to check the integrity of the RNA.

This is anyway we are going to discuss when we are going to talk about northern blotting. So that time we are going to discuss how you can run the RNA gels and how you can test whether the RNA quality is good or not. The RNA isolation by the triazole method shows that after adding chloroform and centrifugation, the mixture separates into three phases: the upper phase, the aqueous phase containing the RNA; the interface containing the cell debris; and the lower phase, the organic phase containing the protein and the liquid. We have actually prepared very small demo clips where we are going to show you how you can isolate the RNA with the help of the Trizol method, and here the students have isolated the RNA from the bacterial cells. But you can actually follow similar steps even with mammalian cells or tissue.

As I said, you know, when we are going to make a deal with the tissue, you are actually going to homogenize the tissue so that you can get a single-cell suspension. So I hope this video, or the demo video, is going to be useful for you to advance your work. Today we will be learning about RNA isolation from the bacterial culture. As you can see, this is a bacterial suspension that has already been prepared. This is a suspension of Staphylococcus aureus, and we have already aliquoted.

So for RNA isolation, we will need around 800 mL. We have already aliquoted in the Eppendorf tubes. So to do that, we need a laminar flow so that the contaminants don't get out. So, as you already know, we have already allocated around 800 mL of bacterial suspension. So we will be performing RNA isolation from this bacterial suspension today. RNA isolation is a very tricky step because it is easily degradable in the environment.

So we have already given the UV for the whole hood. We have cleaned the pipettes with 70% ethanol, and all the tips and everything have been UV irradiated before use. So, a protocol for the RNA solution can be done in three simple steps: one is to homogenize the bacterial cells to extract the RNA from them, then to precipitate the RNA, and finally to purify the RNA. So the first step we'll do is the homogenization of the RNA. To do that, we'll be using the tri reagent; this reagent is basically a triazole, which contains guanidine thiocyanate and phenol.

It inhibits the RNA's activity so that the RNA is not degraded in the system. Now the protocol is that we will be adding around 160 microliters of triazole to the suspension culture. The cap should be put down in the laminar flow hood as always. And this triazole reagent will help to homogenize as well as protect the integrity of the RNA in the suspension. To homogenize, everything is very simple. We have to pipette in and out faster so that the bacterial cells are homogenized; as you can see in this step, I am pipetting a little bit vigorously in and out.

After the thing is done, we will be adding chloroform, which will be around one fifth of the total volume. Earlier, it was 800 microliters, and we have added around 160 more, so it is now around 950 microliters. Therefore, one fifth of the volume will be chloroform. So chloroform will do one thing.

It will help in separating the phases in the mixture as such. So now we will be adding chloroform to the triazole mixture we have already prepared. So, we have allocated chloroform in this reagent bottle. So we will be adding around one-fifth of the volume, which is around 32 microliters. So, as I told you before, chloroform will help in separating the phases.

Actually, triazole is very helpful. By using triazole, we can separate all three components: DNA, RNA, and protein, as you will see in the subsequent experiment. So after adding the chloroform, what we will do is we will tilt a little bit, very gently. It will not be harsh. We will tilt it a little bit and then leave it for incubation for two to three minutes at room temperature.

So I will just put it here and wait for two to three minutes. So after that, we will be putting it in the centrifuge. We have to centrifuge it at 12000 rpm for 4 degrees for 15 minutes. So that phase separation might happen. So I have already put it in the centrifuge itself, and I will start it now.

So it will run for 15 minutes, and then we will get back. So, as you can see, the RNA is just now going to stop. So we'll take it out and then process it further. So after we take out, we'll see the layers being formed. So after centrifugation, you can see very clearly that three types of layers are formed.

This is an aqueous phase that contains the RNA. A white type of layer you see; this contains DNA. And the pink layer, if you see it, contains the protein. So from triazole, we can isolate all three: DNA, RNA, and protein together, but for today's experiment, we will be doing RNA isolation. So we will be taking out the aqueous layer, which is on the top. We have to be really careful not to take out the interface or the bottom layer, so we will be allocating this in the new centrifuge very, very carefully.

We are now elevating the aqueous phase in the new Eppendorf tube. So you have to be very careful. So now we actually have to take it very slowly, tilt it a little bit, and slowly pipette it out. Don't just take the interface; that is the whole point. Still, some is left, so I will try to take more out of it.

As you can see, I am tilting it so that I can see clearly where the thing is going. So, it is always safe to not touch the interface. So, as you can see, I have already allocated it and have not touched the interface. And this much RNA is more than enough for our experiment. Technically speaking, we should not talk while we are doing RNA isolation, as aerosols from our conversation might contain some type of RNAs that could degrade the RNA.

So when we are doing RNA isolation, we should not talk much about it. Now we will be adding isopropanol alcohol; this will help to precipitate the RNA from the aqueous phase. So how will we do that? We will be actually centrifuging it after adding equal volumes of isopropanol. So now we are centrifuging it at 10,000 rpm for 10 minutes; this will help precipitate the RNA after the addition of isopropyl alcohol. Now that the centrifuge is almost complete, we will be moving to the last step of the RNA isolation, which is the purification step by adding around 70% ethanol and then centrifuging it again.

So now we have centrifuged it after the addition of isopropanol. Here, as you can see, the pellet is a little loose, and it is also a bit shaky. That might be due to the lesser suspension culture. I think the bacteria were not enough to give a very big thick pellet, but still, the

pellet is there. So now we have to very carefully remove the isopropanol and add 70% alcohol, which we have already prepared. So, for the 70% alcohol preparation, we have used Merck grade ethanol and double-distilled autoclave water, which is filtered using 0.

2 micrometer membrane filters. So let us remove this isopropanol first. You have to remove it very slowly so as not to take the pellet out, and if you are not sure whether the pellet will come out or not, you can leave it and then take a small pipette again. for example i am using a p1000 which is a grading of 100 to 1000 so for now i will leave this pipette and then i will take another pipette which is of a lower volume so that i can slowly slowly take out the isopropanol So this is a pipette of 20 to 200 microliters, so this would be the best fit for my needs. Now, I will be setting it to around 100 microliters; it's a lower volume, and we can take it slowly. Now we have taken out the isopropanol, and we will be adding 70% ethanol.

We have to resuspend the pellet, so what we will do is add around 1 ml; you can add 1 ml or 500 ml. This is a washing step and a purifying step for RNA. After you add, you have to pipette in and out a little bit so that the things are resuspended, and after that, we will go ahead with the last centrifugation, which is 10,000 rpm for 10 minutes at 4°C. So I am adding around 1 ml; it is done, so we will go ahead with the centrifuge. Now we are performing the last centrifugation step of the entire protocol.

We will be doing 10,000 rpm for 10 minutes at 4°C, and this will be helpful for washing the RNA. Now we have centrifuged the last step, which was after the addition of 70% ethanol. So the next step is to air dry, remove the whole ethanol, and air dry it. And after air drying it for 10 to 15 minutes, we will add RNA's water so that we can resuspend the pellet in it, so let's take out the ethanol. So after this step has been done, what we can do is, because the pellet is there, we can just tilt it a little bit so that the residual ethanol will come out.

Now we will keep it for air-drying. Now, after 15 minutes, the thing is fully dried, so we will be adding the RNS-free water. This water we have already; I showed you before we made the 70% ethanol, so we will be adding around 50 microliters to it and then resuspending the pellet again.

So just add. and we suspend it a little bit. Don't be too harsh. Pipe it a little bit, and then just leave it. So the next step will be to quantify this RNA. First, we will quantify it using a NanoDrop, and secondly, we will visualize the RNA using gel electrophoresis. So, the RNA can be stored at minus 20 degrees centigrade. We should not store it at 4 degrees for a short time.

So, I will be storing this at minus 20 degrees centigrade until further experiments are done on this. Now, today we will do RNA quantification. So, this is an instrument. This is a nanodrop machine.

So, this will measure the amount of RNA in one microliter. So, we will go to the nucleic acid section. As you can see in the nucleic acid section, there are a lot of assays that can do protein lust everything, so this is the whole template; here you will see the concentration. This is A260x280; reading a peak will come if the RNA is there, so NOT is set for DSDNA, which means double-stranded DNA. What we will do is set for RNA. And now we have clicked RNA; as you can see, we will proceed with the blank addition to measure blank.

First, what we will do, as you can see here, is this portion where we load our sample area. So we will first clean it nicely on both sides, wipe it with a lint-free wipe, and after that, we will add RNA-free water where we have already suspended our RNA, adding exactly 1 microliter. So here, addition is to be done on the red dot, as you can see here. I will be adding directly here for RNA quantification. Now that the whole sample has been loaded, I will close it and select blank, as you can see here. So I'll click on blank now, and it will adjust the blank reading first, and then after that, we have to load our sample.

So now that the blank has been set, we will load our sample after that. To do that, we have to first wipe here again because we have already added, and then what we have to do is take our RNA, whatever we have made that day, and Just add it, close it, and say, "Sample this green one; this is a sample." So we'll just click it here so it will measure the RNA content, the concentration of nanograms per microliter.

So, as you can see, the RNA concentration is 556.52; the 8 to 60 by 20 reading is 2.23, which is a very good reading. There is no contamination; a nice peak can be seen at 260, so it's a very good amount of RNA with no contamination of protein and DNA. So now I'm sure you have seen the demo video, and this demo video could be very useful for you to replicate these steps in your laboratory. Although we have shown the steps with the help of a simple system like the prokaryotic system, it can also be replicated with other types of cells. You can use mammalian cells, yeast, different types of tissue materials, or even bacterial cells.

The first step is where you are actually going to prepare the cell lysate. After that, the subsequent steps are going to remain identical, whether it is a prokaryotic cell or a eukaryotic cell. Now, once you isolate the RNA, right, you are actually going to have the first question: what will be the concentration of this RNA? and whether the quality of this RNA is good or not. Just remember that when we were talking about the DNA, we also

asked the same thing, right? So in the next step, we are going to talk about the RNA, including both the concentration and the purity. So the purity of the RNA can be detected in the same way that RNA also absorbs very strongly at 260 nanometers.

So RNA is actually, what you can do is take the absorbance at 260 nanometers. And once you measure the RNA at 260 nanometers, it will actually give you the values. So if there is, you know, pure RNA, right, if it is pure RNA, it is actually going to have a very specific absorbance at 260 nanometers. If it has protein contamination, then the ratio of 260 to 280 will actually vary among the RNA species. So, it is actually going to have the, if it is pure RNA, the 260 by 280 would be around 2, right? Because RNA absorbs very strongly at 260 nanometers, if it has protein contamination, then it will actually have a level of less than 2, right? That's how you can know the purity of the system.

Or you can have the purity of RNA. Now the next question is how you can do the estimations. So the estimation can be done at the absorbance level. So you can take the absorbance at 260 nanometers, and you will be able to use the formula to determine the concentrations. Or you can actually use the method. RNA estimation by the orcinol method. Remember that when we were talking about the DNA estimations, we discussed the DPA method, right? So, what is the principle? This is a general reaction for pentosins and depends on the formation of peripheral.

When the pentose is heated with concentrated hydrochloric acid, it reacts with furfural in the presence of ferric chloride as a catalyst to give a green color that can be measured at 660 nanometers. So, what you are going to do is RNA; RNA is ribonucleic acid, right? Which means it's going to have ribose as a sugar, right? Remember that for the DNA, it is the D of the ribose, and that's why, when you hit this RNA in the presence of the SCL, it is actually going to form the furfural, right? And the furfural is actually going to react with the arsenol. And it's going to give you the green-colored compound or solution.

And that actually is going to have the lambda max at 665 nanometers. Okay, so this is 665 nanometers. So what you can do is actually create a calibration curve with the lamb absorbance at 665 nanometers versus the RNA, right? So you can actually take the different concentrations of RNA, and that's how it is going to give you the calibration curve. And then you can actually run the same with the unknown samples; suppose this is the absorbance of the unknown samples, and then you can actually determine the concentration of the RNA. So what are the things you require? You need the standard RNA solutions, and you require the 0.

2 mg per ml in one percent per chloric acid or the saline buffer. Then you also require the arsenal reagents so you can dissolve 0.1 gram of ferric chloride in 100 ml of concentrated

HCl and add 3.5 ml of 6% weight by volume arsenal in the alcohol. And then you also require the buffered cell line so you can actually make the NaCl, and you can make the shifted buffer PS7, so it's very simple; you don't require a lot of agents.

Also, the procedure allows you to prepare the different concentrations or amounts of RNA, such as 0.2, 0.4, 0.6, 0.8, and 1 ml, into a series of labeled test tubes. Then you pipette 1 ml of the given sample into another test tube. Make up the volume to 1 mL in all the test tubes. The test tube with 1 ml of distilled water serves as a blank, right? So that is the blank reaction so that you know what the background absorbance of the arsenol reagent itself will be.

Then you add 2 ml of ethanol reagent to all the test tubes, including the test tube labeled as the blank and the unknowns. Mix the content of the tube by vortexing or shaking the tube and heat in a boiling water bath for 20 minutes. So this step, and then cool the content and record the absorbance at 660 nanometers against the black, right? Then plot the standard curve by taking the absorbance concentration of the RNA along the x-axis and the absorbance at 65 on the y-axis. Then, from this standard curve, calculate the concentration of the RNA in a given sample.

This is the table that you are going to use, so from the standard RNA stock, you're going to have the 0, 0.2, 0.4, 0.6, 0.8, and 0.1. Then you add the water to make up the volume to 1 ml, so the total volume of the RNA and the water will be 1 ml. For example, in this case, you don't have the RNA. So you are only going to take 1 ml of water; in this case, you have 0.

8, so you are going to add 0.2, so the total is actually going to be 1 ml. And then take this table, and for the unknown, you are going to take one ml of unknown. You can take the other values of unknown also, but accordingly, you are going to add the water, and then you are going to add the volume of reagents. So you're going to add 2 ml of the Orsenol reagent, which you will prepare with the help of ferric chloride, HCl, and Orsenol. And then you incubate this in a boiling water bath for 20 minutes, cool it down, and then take the absorbance.

So the absorbance you are going to get for the 0 RNA is going to be treated as the blank. And this has to be subtracted; although you're not going to get the zero values, you are going to subtract that value. So it's going to be zero. And then you're going to have some values for other considerations and for the unknown as well. Using these values, you will be able to draw a calibration curve.

So what you're going to do is have the absorbance at 665 versus the RNA concentrations.

Which means the microgram. And then you are going to have the standard curve. So with the help of the standard curve, you can determine the concentration of the unknown RNA species. And if you want to read more about the RNA estimations and other topics, you can refer to this Plummer's book. This is a very interesting book that deals with the practical aspects of biochemistry.

And it's very interesting because it gives you the steps on how you can follow and how you can prepare the recipes and so on. So, this is all about the RNA. What we have discussed is the structure of messenger RNA, the different parts of RNA, and their functions. And then we have also discussed how you can isolate the RNA and how you can verify the RNA with the help of the estimations and the purity of the RNA. So, with this, I would like to conclude my lecture here. Thank you.