Bioengineering: An Interface with Biology and Medicine
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Lecture - 13
DNA Tools & Biotechnology-V

So now we will see how we can study the expression pattern of the genes in a laboratory setup. The cDNA synthesis describes generation of complementary DNA or the cDNA from RNA template by the process of reverse transcription. The cDNA which can be used directly as template for the polymerase chain reaction. It can be amplified using enzyme reverse transcriptase.

So in a way we can actually measure the cDNA and access the levels of expression of a gene. So let us start a laboratory demonstration now in which you can see how to make cDNA and then use that for amplification using polymerase chain reaction.

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Hi, I am Swati and I am one of the (()) (01:09) for this course. So today I will be explaining the first few steps that are involved in cloning of a human eukaryotic gene. Unlike DNA the first step in the cloning procedure is isolating RNA from human whole blood. Unlike DNA RNA is a very unstable biomolecule.

And therefore all the before beginning any RNA extraction experiment the surface needs to be cleaned with this chemical which is known as RNaseZAP which will prevent the RNA from degrading.

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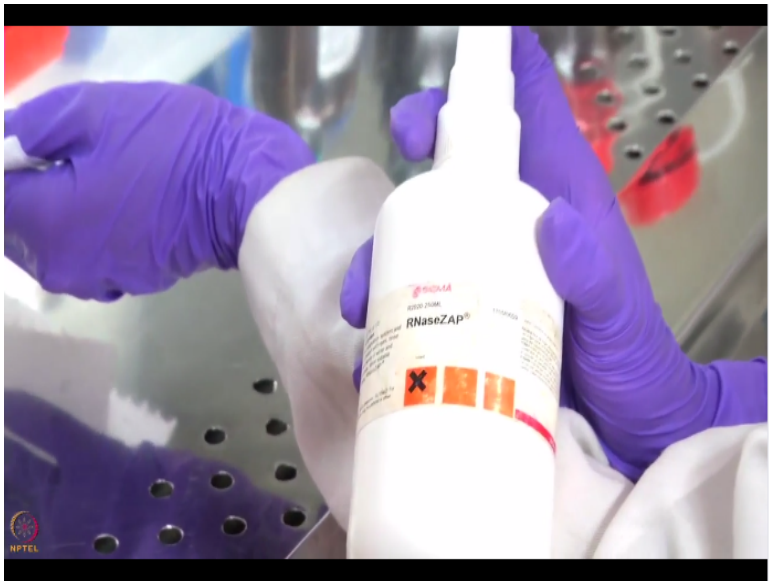

So we will first clean the surface with RNaseZAP as well as all the surface which will be touching any of the tubes or pipettes etc. So the protocol that we will be using today for extracting RNA is the phenol chloroform protocol.

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The reagent that will be using is called TRIzol which is a commercially available reagent. So we will be taking around 200 microliter of whole blood.

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After adding the blood, we will then be adding the TRIzol reagent. So that TRIzol reagent basically contains phenol and guanidine thiocyanate, which will help to lyse the cells and bring the cellular contents into the solution. This is then mixed thoroughly. After addition of TRIzol the tubes are mixed nicely and then centrifuged.

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After addition of TRIzol and mixing the tubes well, we will now add chloroform to the tube.

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So once the RNA has been isolated and quantified, the next step is conversion of this RNA into the complementary cDNA strand. So for this the first step involves so during the RNA extraction procedure, there are high chances of the isolated RNA having genomic DNA contamination and therefore the first step in the cDNA synthesis involves removal of genomic DNA contamination.

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So for this we will take around 1 microgram of the quantified and extracted RNA and then add the enzyme DNase. So DNase is the enzyme that will degrade any of the genomic DNA contamination that might be present in the extracted RNA sample. So this is the DNase that we will be adding.

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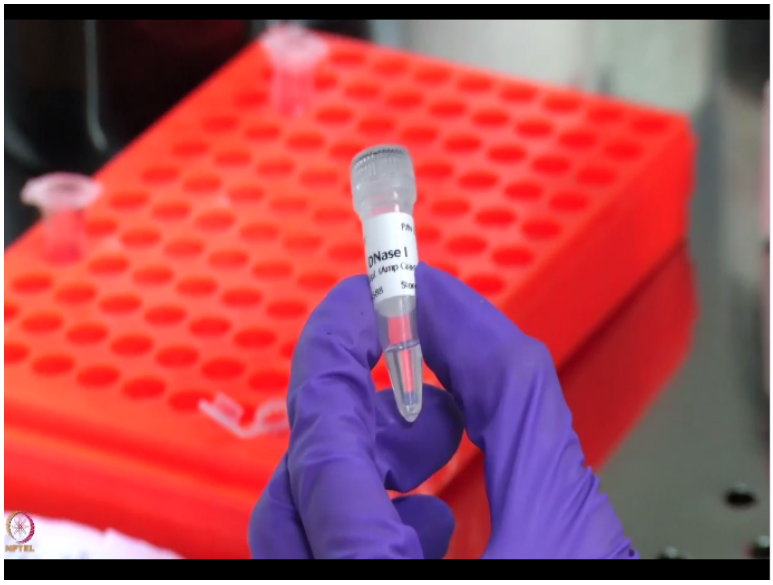

We will then add a buffer which is compatible with the DNase enzyme and which will increase its activity. This reaction is then incubated at 37 degree Celsius in the dry bath for 30 minutes.

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So after addition of chloroform and centrifuging the tubes we will now see that an aqueous layer has formed as you can see.

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After incubating the samples overnight in isopropanol, the samples were centrifuged at high speed. Centrifugation at high speed will result in the pelleting down of the extracted RNA. The pellet formed will is then dissolves in sterile distilled water as you can see. The next step after RNA extraction is the quantification of the extracted RNA. So the quantification method that we would be using is a spectrophotometric reading at 260 nanometers.

And for this we will be using the uDrop plate. First the plate has to be cleaned with ethanol.

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Since the RNA was dissolved in distilled water, we would be using distilled water as a blank. So we would be taking 2 microliter of sterile distilled water and then taking 2 microliter of our extracted RNA which is dissolved in water. The advantage of using the micro drop plate is that very small amounts of your RNA solution is needed to take the readings. We will now

place the uDrop plate in the Multiskan GO instrument in order to take readings at 260 and 280 nanometers.

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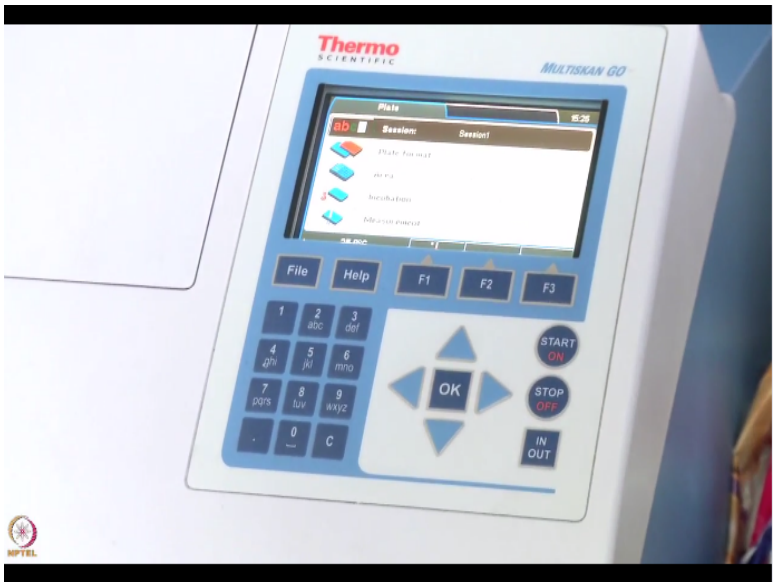

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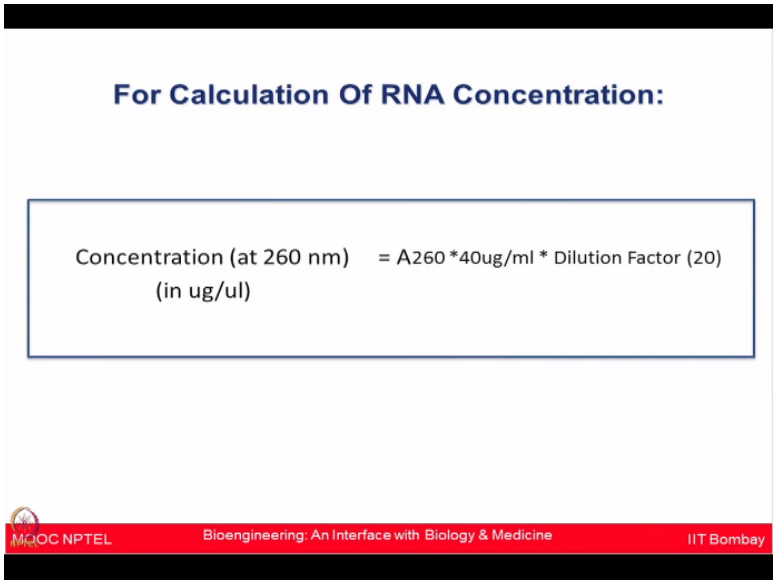

After taking the absorbance at 260 nanometers, so this is the formula that we will be using in order to calculate the concentration of the extracted RNA and absorbance reading of 1 at 260 nanometers corresponds to around 40 microgram per ml of single stranded RNA. Therefore, in order to get the concentration of R sample, we will be multiplying the A260 absorbance reading by 40 microgram by 40 which is the standard as 1 corresponds to 40 microgram divide and multiplying it by the factor.

In this case, for this particular instrument the factor is 20. After incubating the tubes at 37 degree Celsius with DNase we will now add 1 microliter of 50 millimolar EDTA. EDTA will basically sequester all the divalent cations and prevent the RNA from hydrolysing while heating at high temperatures. So we will now add 1 microliter of 50 millimolar EDTA to our tube.

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After the EDTA step we are now done with the step of genomic DNA removal. We will now proceed with the cDNA synthesis step. So the cDNA synthesis step involves the addition of the following components.

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We have the OligodT primer, reaction buffer, the RiboLock RNase inhibitor. We have 10 millimolar dNTP and the reverse transcriptase enzyme. Since we are using an mRNA as a

template, we are going to be using an OligodT primer, so this is nothing but a stretch of time in nucleotides which will go and bind to the poly A tail of the mRNA. We will now add these components to the tube in the following order.

So we are adding the OligodT primer as I said which will bind to the poly A tail of the mRNA.

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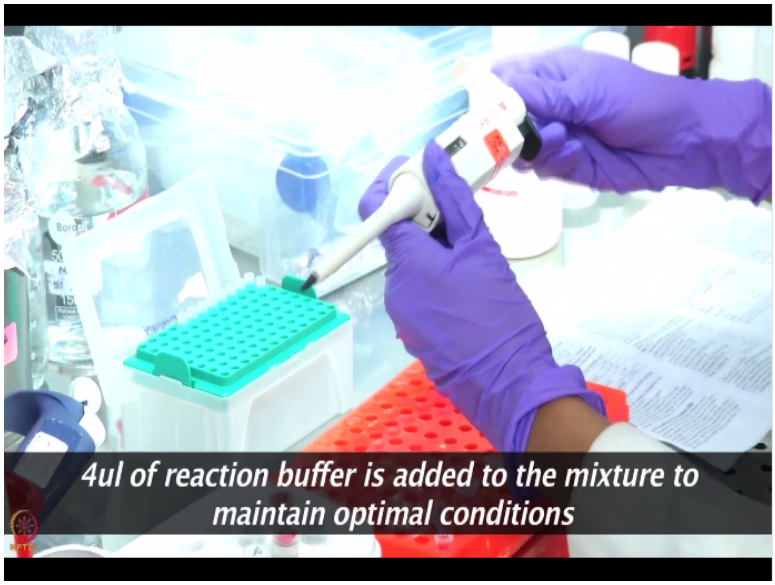

We will now add 4 microliter of the reaction buffer. We will then add the RiboLock RNase inhibitor. Then this will basically prevent RNase activity which will degrade which is the enzyme that can degrade our RNA. We will then add 10 millimolar dNTP and finally we will add the enzyme reverse transcriptase. So this is the enzyme that will convert our RNA template into cDNA.

As with any experiment, controls are very important and for cDNA synthesis we have 3 important control reactions that are to be set along with your sample of interest. The first is the no reverse transcriptase control. The no reverse transcriptase control will have all the components as described previously which will include the primer, the reaction buffer, the RiboLock RNase inhibitor, the dNTP's, your genomic DNA removed RNA template and will only not have the reverse transcriptase enzyme.

This control will basically test for any genomic DNA contamination that can be present in our samples. The second control reaction is a no template control.

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The no template control basically will have all the components of the cDNA synthesis reaction except your template DNA. This control will test for any contamination that is present in any of the reagents that are used for the cDNA synthesis. The third control is the positive control with the cDNA kit that we are using.

We also get a control RNA, so this reaction will have all the components of the reaction mixture and instead of adding our sample as the template we will be using the control RNA as a template. After addition of 50 millimolar EDTA, the tubes will then incubated at 65 degrees for 5 minutes.

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After setting up the control as well as sample reactions, we will now be incubating sample tube, the no reverse transcriptase control, no template control and the positive control tubes at 42 degrees for 1 hour.

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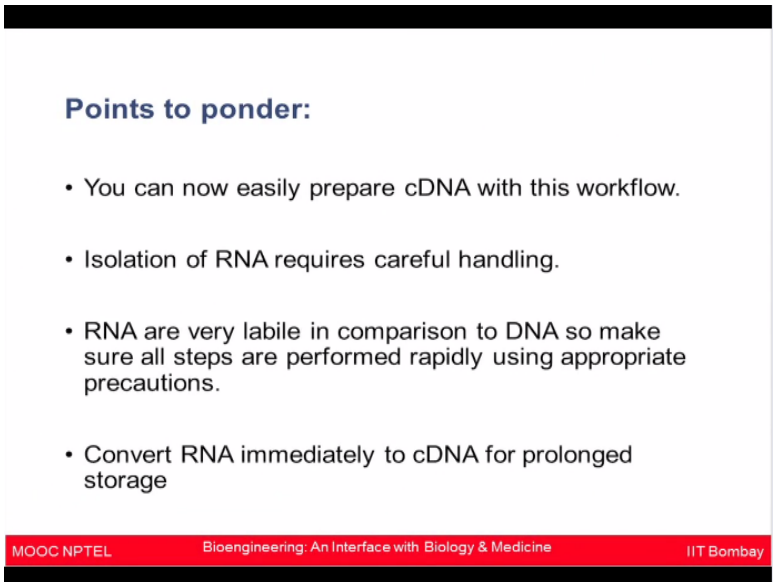

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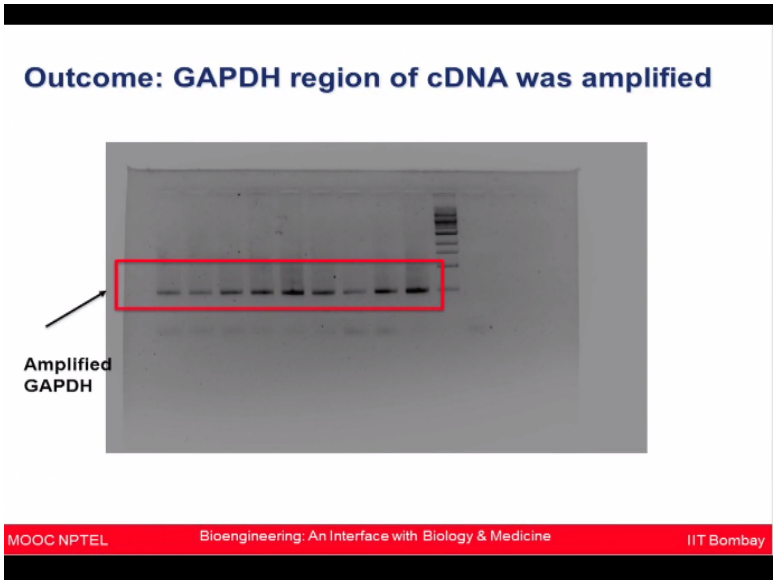

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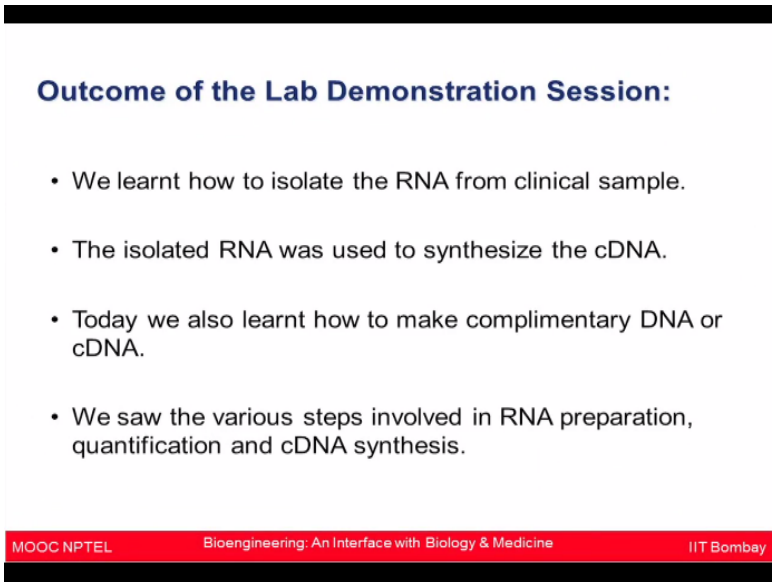

I hope you got a glimpse of how expression studies can be done in a laboratory setup. Today, we also learnt the various precautions which need to be taken while handling RNA which are labile molecules for successful conversion to the cDNA. Once you have made the cDNA molecule, you can store them for long time and then you can use them for PCR when you are ready to do those reactions.

I hope this was a useful lab demonstration. We will continue our more concepts and discussions in the next class. Thank you.