Genetic Engineering: Theory and Applications
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Genetic Engineering: Theory and Applications
Module 7 Isolation and Purification of Product part 1
Lecture 20 Basics of Chromatography part 2

Hello everyone this is Doctor Vishal Trivedi from departments of bioscience and bioengineering IIT Guwahati.

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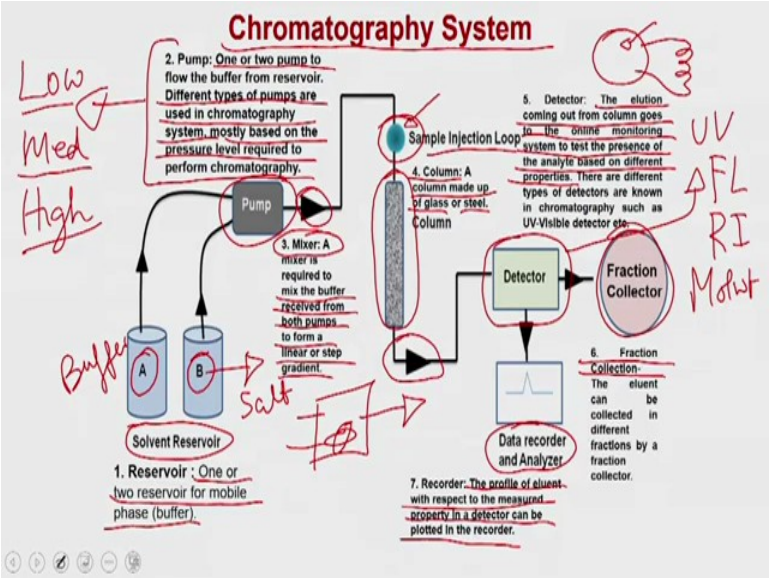

Now in a chromatography system which is actually a integrated system where you have the multiple components put it into a machine and that can be used to enhance the efficiency as well as it can improve the purification. So it has multiple components you have a solvent reservoir. So the component number 1 is solvent reservoir ideally you can have 1 or 2 reservoirs in some cases in some of the machines you can still you can have even 4 reservoirs.

So the purpose of having the 2 reservoir is that the first reservoir can be used to equilibrate the column whereas the other reservoir can be used to generate a gradient. So in that case for example you can fill the A with the buffer and the B you can use and fill it with the salt and that is how if you want you can actually make a gradient between A and B with the, by the mixing these 2 you can be able to make a gradient of this salt and that can be used for the chromatography technics.

Now these 2 reservoirs are attached to the pump, the pump you can have 1or 2 pump which means you can have the pumps dedicated to A you can have the pump which is dedicated to B different types of pump are people are using in chromatography system for example you can use the peristaltic pumps you can use the syringe pump or you can use the infusion pumps and in some cases you can use the high power and high pressure pumps.

Mostly these pumps are based on the pressure level required to perform the chromatography and accordingly we can use the chromatography at low pressure as well as the high pressure. So based on the type of pump which you use in the chromatography the chromatography could of low pressure chromatography where you will use the peristaltic pumps or you can have the middle pressure chromatography where you will use the syringe pump or the fusion pumps or you can use the high pressure chromatography or high pressure pumps where you will use the pumps which are made up of steels.

Now after the pumps you have the mixture chamber the purpose of the mixture is to mix the buffer received from the both the pumps to form a linear or a step gradient. So the mixer is nothing but a kind of a magnetic starrier. So in the mixer what you have is a chamber which actually has a beed and as soon as it receives the liquid from the 2 chamber or reservoir it actually mix them in a, such a way that it actually makes the linear or the step gradient.

And then this liquid can be delivered into the column and can be used for the different type of chromatography technics as per your requirement you can have the linear gradient either the upward linear gradient or the downward linear gradient. Now mixed with the mixture you have the sample injection port sample injection port allows you to inject your sample. So in most of these chromatography system you cannot be able to inject the material directly into the column.

You can use this sample injection loop and the sample injection loop is nothing but actually a ball where you can use a syringe to inject the sample and that sample actually goes and fill into the loop and then when you give the amount to the system it actually loads this material into the column. Now next to that sample injection loop you have a column, a column could be made up of glass or steel.

So depending on whether you are using the low pressure column or middle pressure chromatography system or high pressure chromatography system you have the option of using the column which is made up of glass or to the steel. Now next to the column now

chromatography is over the sample is going to come out and then it will be analysed by a detector.

So the next is detector in the detector it is actually a online monitoring system. So the illusion coming out from the column goes to the online monitoring system to taste the presence of analyse based on the different properties. So the detector, detector could be UV visible detector, so you can put a UV visible spectrophotometers so you can have the UV visible detectors you can have the fluorescence detector.

So that actually will measure the fluorescence of the molecule which is coming out from the column or you can use the RI detector that will actually is going to measure the reflective index or you can use the molecular weight or you can use the (())(05:52) as a system. So in that case this machine is going to be called as the LCMS because it is actually going to do the chromatography and at the end what you are going to see is the molecular weight of the molecules and so on.

So you have the choices of different types of detector which you can put and that detector is going to detect that particular physical property of that particular molecule which is coming out from the column and then the detector will give that signal to the data recorder and analyser. So in the data recorder and analyser you will see the profile of the eluent with respect to the measured property in a detector and it can be plotted in a recorder.

This recording only we have called as the chromatogram. So the property is being plotted against the elution volume or the elution time and that you can actually monitor in a online system. So you will see as long as the protein is coming out you can actually be able to collect the fractions. Now this detector is also collected to the fraction collector. So as soon as you will see that some compound or some product is coming out you can actually ask the machine to collect that particular fraction and that will be collected into the fraction collector.

So in the fraction collector you have the different types of glass tubes which are being kept and these glass kept tubes are allowed you to collect that particular fraction or that particular protein into a separate tubes. Now this is all about theoretical explanation of chromatography system but when you see a chromatography system in your laboratory or when you see a chromatography system the operation of these chromatography system is very very different from the description just I now I have given.

So for this purpose we have prepared a small demonstration of the chromatography system which is present in our laboratory and the help of this demonstration we are going to show you how to operate the chromatography system how to connect the column how to connect the loops how to injects the samples, how to and as you know that most of this chromatography systems are being operated through a interface through computer.

So, you can also use different types of soft wares to operate the machine as well. So, with all this we have shown into a small demo clips. In this video we will show you how to operate (())(8:38) instrument and a basic principle laying mechanism of separation of the protein using your HTLC. HTLC ranking but fast protein liquid chromatography. We can say it is a derivatization of HTLC.

The main difference between HTLC and FPLC is FPLC can only be used for the separation of the proteins and sometimes small small (())(09:09) also it will have columns available but in HTLC we can use (())(09:17) separation of small molecules, suppose which you have in your chamber that also thing can be separated using HTLC. The column what we will use for HTLC and FPLC are (())(09:34).

In case of FPLC we will use plastic columns but in case of HTLC columns we will use steel columns, because the main reason when we are using like this is if you use stainless steel column in FPLC because of the salts and high concentration of the salts and different material we are using (())(10:06) the steel so that is why there may be improper separation of the (()) (10:15) which we want to separate.

So that is why we will use a poly plastic column will (())(10:22). The separation of the proteins grow HTLC is based on the size and shape of the protein. So if you have like in gel filtration in chromatography or size exclusion chromatography in all those mechanicals like size exclusion gel filtration can be applicable in FPLC also. So it depends on what column you using.

So if you want to purify (())(10:53) proteins you can use nickel nta column, pre packed column, suppose if you want to only separate hydro low molecular protein you can use any gel filtration column suitable for your protein but the (())(11:10) behind the separation is same. So this details of like size exclusion or gel filtration we have shown in previous video. In this video we will show how this system can be operated.

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We are on our lab we are using actor loop this is from G health sciences. So all the complaint whatever we show it is similar in other instrument in other company system also but only the I have picture of the instrument changes. So let us see, what are the parts it contains?

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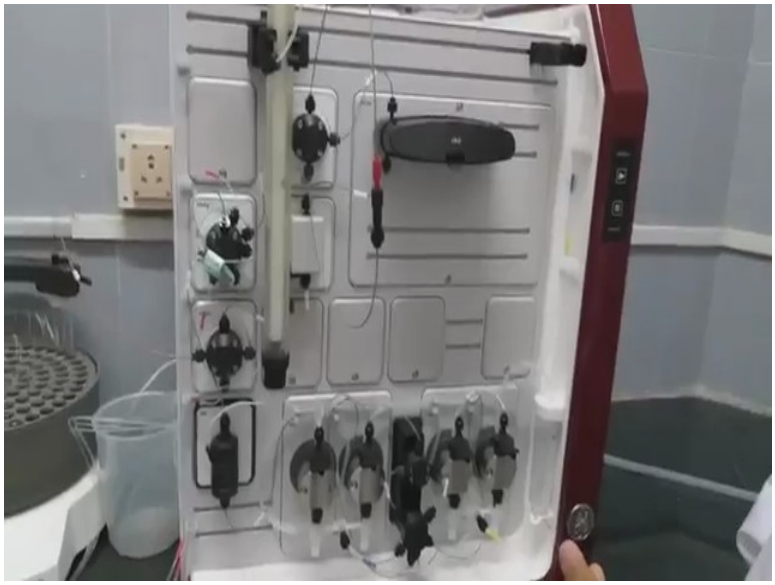

So this is the instrument it is connected to a system for observation purpose.

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So in this system it contains this stationery phase.

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This is the column.

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This is the mobile base that means it buffers this is the area we will keep all the buffers. So it starts with the pumps actually.

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So these two are pumps this one pump and two pump this two sets. Whatever buffers are coming from this buffers tray it will enter here okay these pumps whatever the pressure they are getting they can be embedded here this is the pressure monitor. So once he pressure is monitor it will go to for mixer.

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This is the mixer wave to different purpose suppose if you are using, if you are purifying through nickel nta column. So in that case you will need a middle zone in separate buffer prepared and one is the (())(13:07) pressure buffer. In that case if you want to elute that particular protein that particular hostable protein how to mix the both the buffers A and B for instance.

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So those buffers can be mixed here once the mixing is done it will directly goes to the in let loop.

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So once it entered in let loop it will go to this chamber it can be connected to the column. So the top portion of the column it will connect here we will show you how to connect the column in coming video. So after that whatever it comes it will enter here and it will come directly into the UV chamber were the eluted component will be detected.

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So starting the instrument there is a power button right side of the instrument you have to just turn on the instrument then you can see a white light is blinking here.

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That means the system just started.

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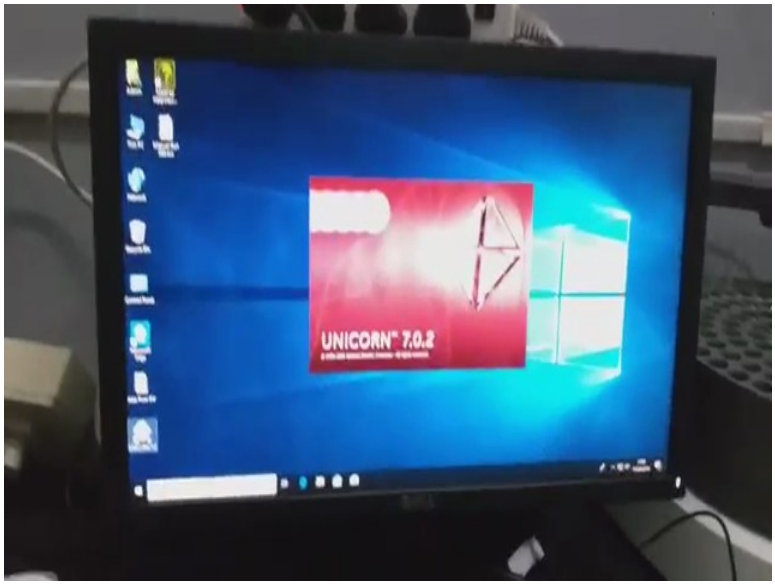

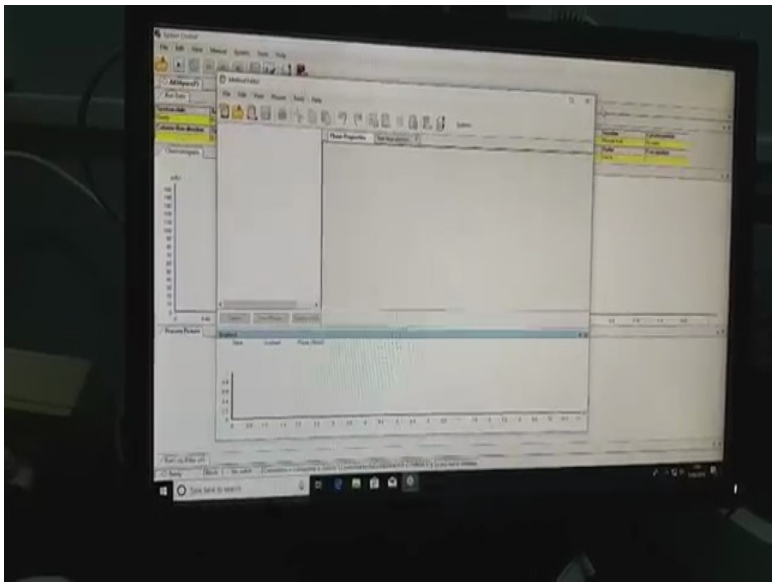

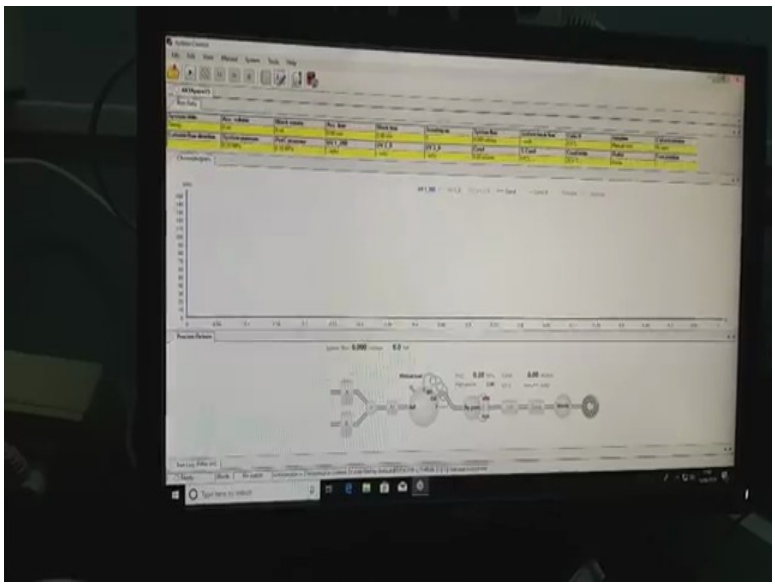

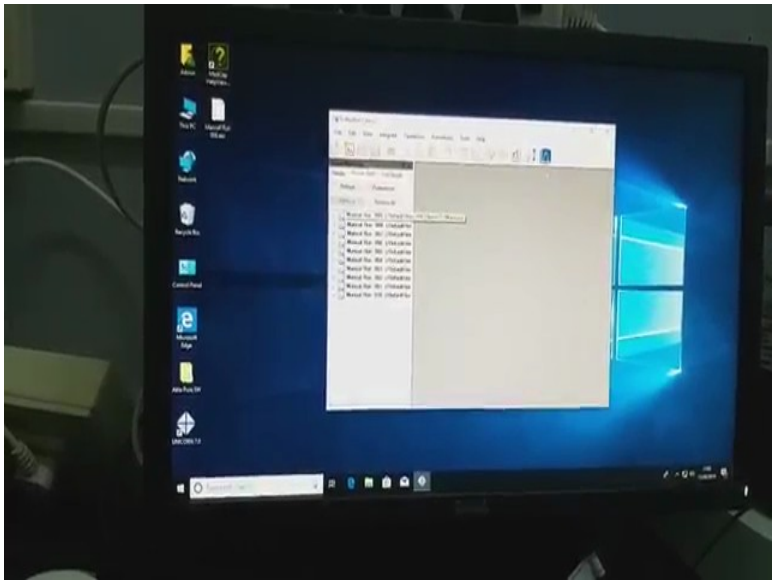

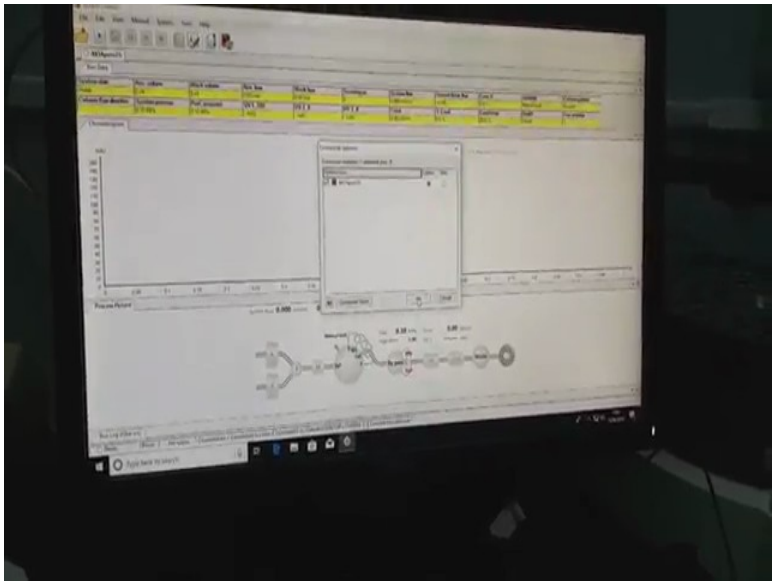

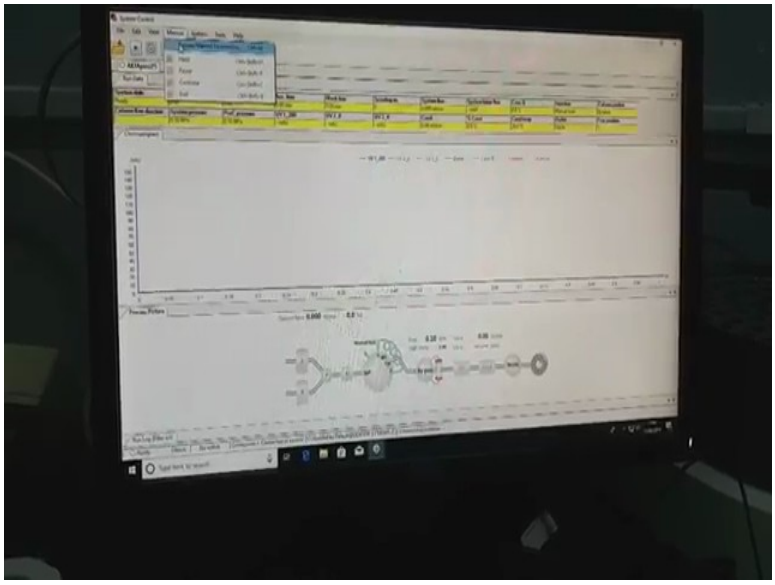

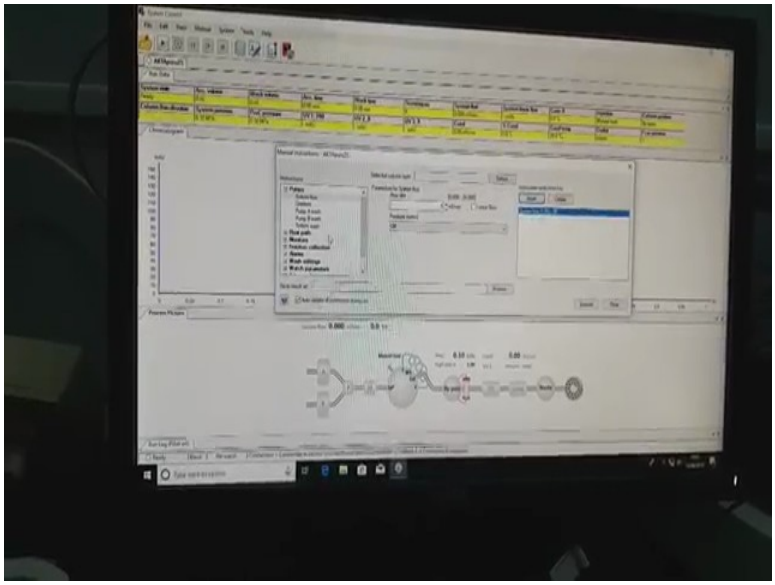

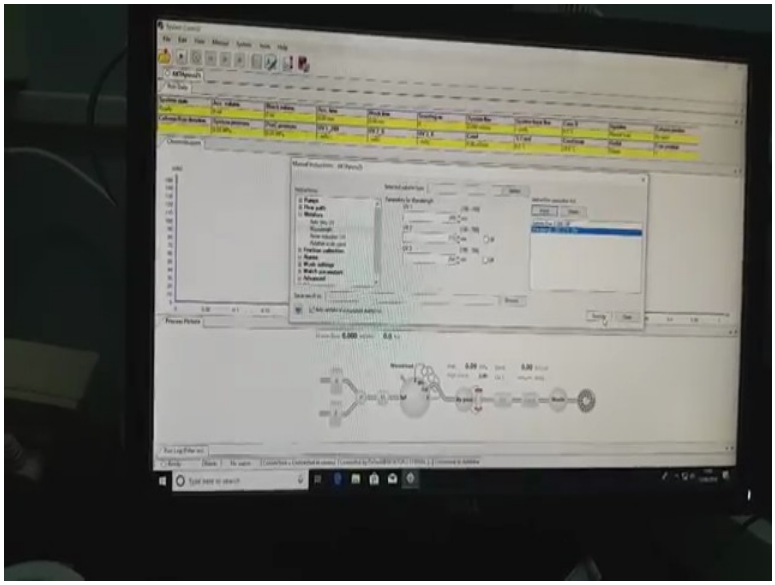

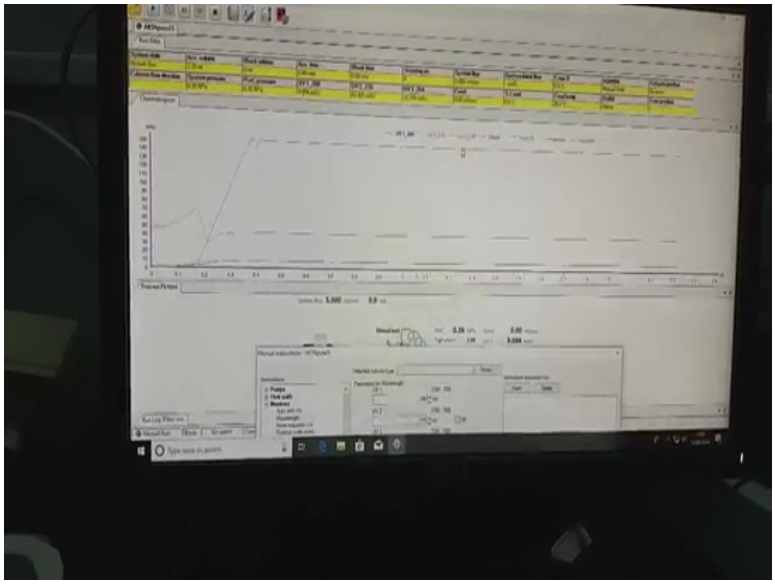

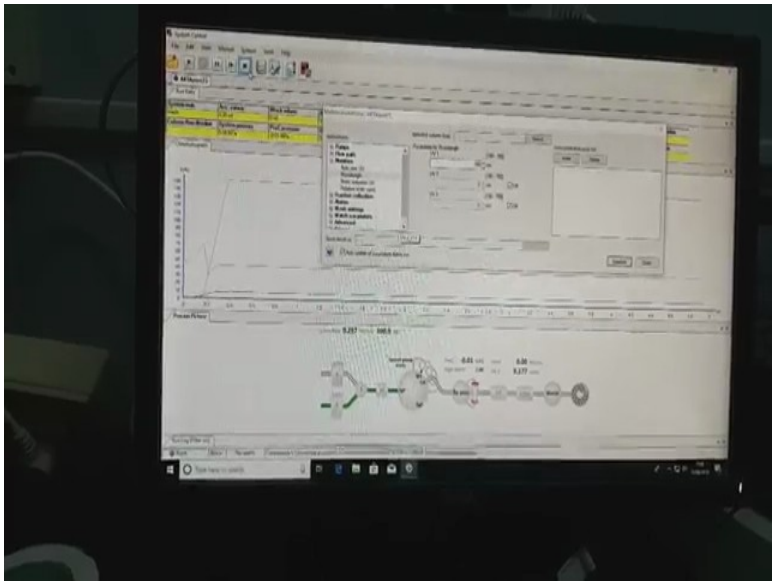

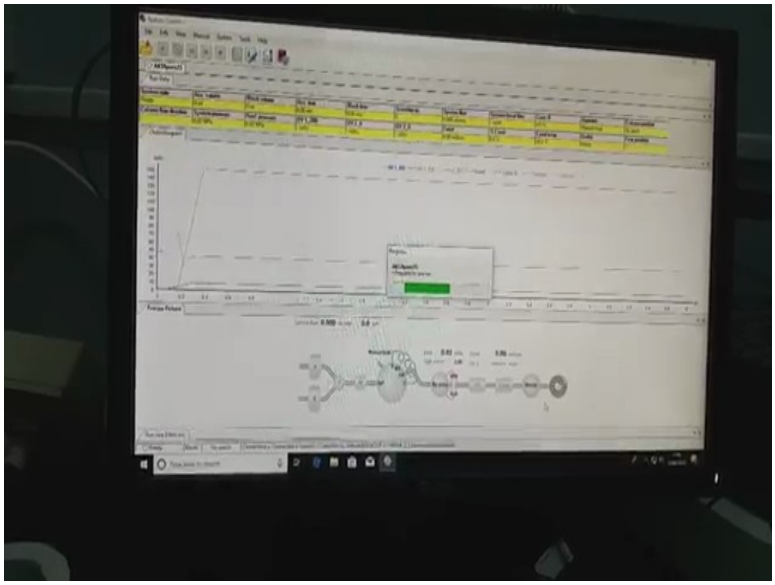

So after that we will go to this software part. For analysis of any elution we can use this it comes with the instruments it is the unicorn software. We use for analysis purpose. So just to double click on that one and it will take you to the software. So it will give you 3 popup windows one is methodetitor another one is system control and evaluation browsing. So this is system editor where you can see chromatograms and the other one is evaluation classic where you can analyse your chromatogram just in go for system control. The first thing we have to do is connect the instrument.

So here you can see the connected instrument tanpura 25. So just say ok it will connect the system. So this will give different we can change different commands using this software just go to manual execute manual. So this is the manual instructions software or dialog box where you can change things7. So here different parameter you can change through this popup window like pumps flow path and various parameter such as monitors just go to pumps, so here you can change the system flow.

So we can keep up to 20 ml if there is no column connected. So normal condition you can keep 5ml also. So you just say inside this thing in order to executed by system. Now here it is monitor very important thing we have 3 wavelengths here we can monitor at 3 different wavelengths. So your choice you can use, so we are giving 280, 259, 254 just insert and say execute.

So it started you can see the green path is highlighted and also chromatograms also appear in the chromatogram area. So it will give 3 different chromatograms, so 1 corresponding should blue that is 280 nanometre for (())(17:37) second one is 254 nanometre for RNA or DNA related and third one is 215 for peptide. So here we can see the path of the flow how it is connected starting from buffer ray so here buffer ray and it will through the pump and mixing through mixer it will go to all the way to waste.

So here different parameter we can change during running we can change B also if you want to change B you just say start pump B. So see we can see highlighted area if you stop the program it will say automatically see you can see some dialog box appear preparing for new runner this is software introduction.

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Now this is this are the buffers we are going to use for this (())(18:40) the buffers will need to be filtered through fine to micron filter and also D gassed for D gassing purpose we will use bath sonicator. So it will remove any air or a bubbles present in the buffers it will removes things. So we are using the most common buffer that is phosphate buffer with having pH 7.4 and this is the (())(19:12) water and this is 20 percentage ethanol all the buffer were filtered through fine to micron filter paper and also D gassed.

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So the washed the system already been the system is being already washed. So now what we will do we will connect the column. So here precautions need to be taken while connecting this thing. So if you have any air bubbles through this loops or the pipping system it will

directly enter into column which will destroy it. So to prevent that we have to make sure all the loops and pumps got washed thoroughly and they will connect in running condition before connecting the column we have to remember few point this column whatever the bits are there this is in 20 percentage ethanol.

So if you directly connect it already ethanol is there whatever the flow rate we are giving it will give more back pressure. So the bit between the, the distance between the column filter and the bits secured bit may increase. So that will reduce efficiency of the column.

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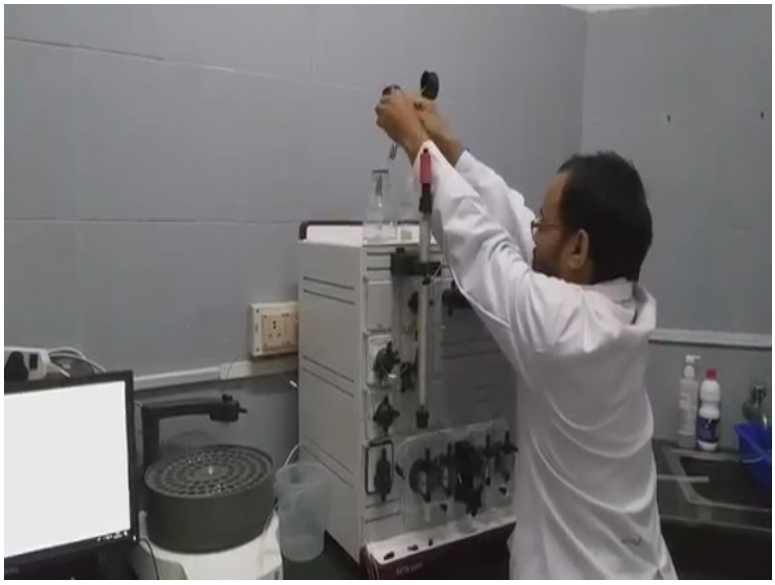

So what we will do we have to change this pumps into water. So we changed into water now we can connect to the column there also some of the precaution needs to be taken if you are using chilled buffers suppose you did cold buffer. So that means you have to bring those all the component of the system to the temperature which we want to use for your purification otherwise if you having chilled buffer which directly enter into column that may (()(21:43) or restate some of the salts present in the buffer inside the column.

So that will also reduce the efficiency. So this is also need to be taken care while running the FPLC. Before continuing the columns, we need to adjust few parameters.

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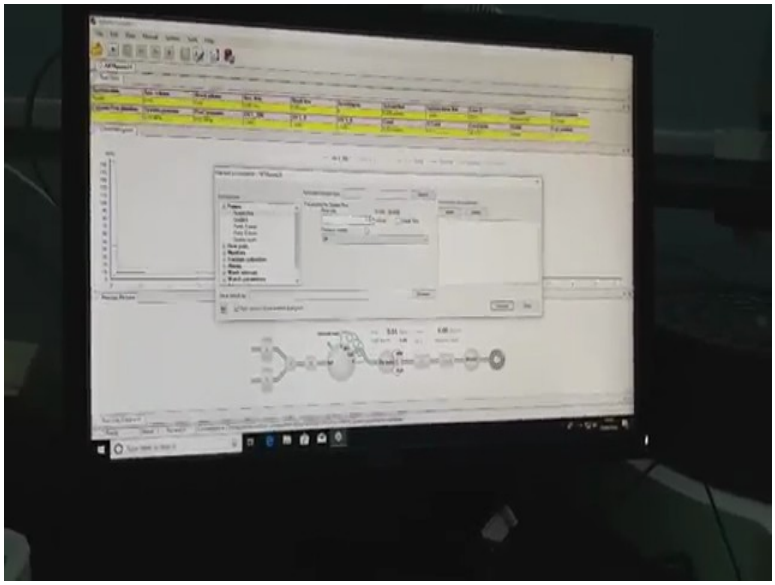

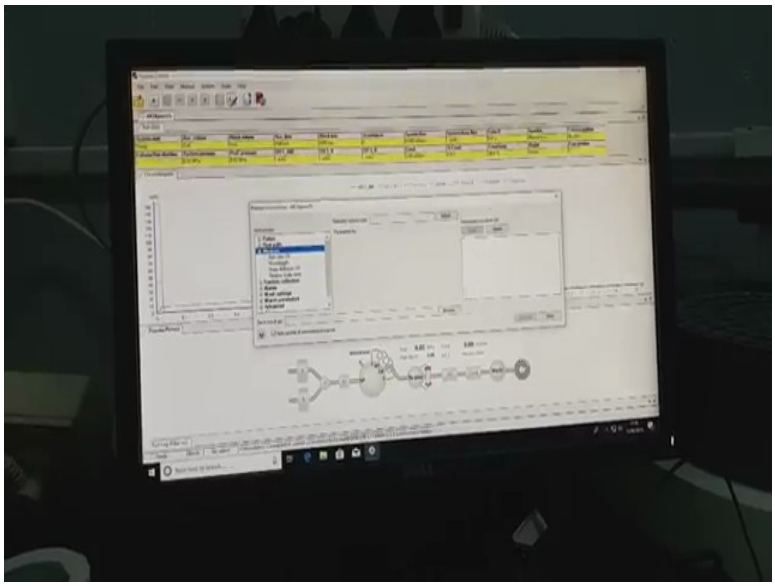

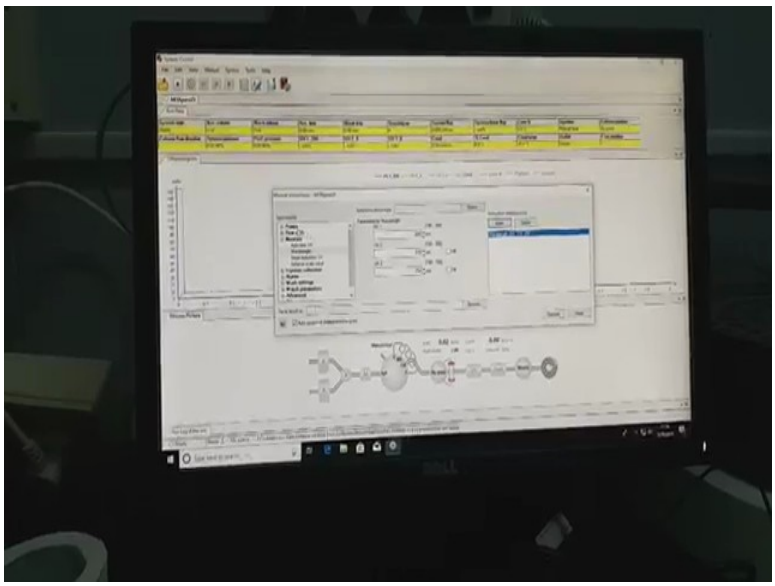

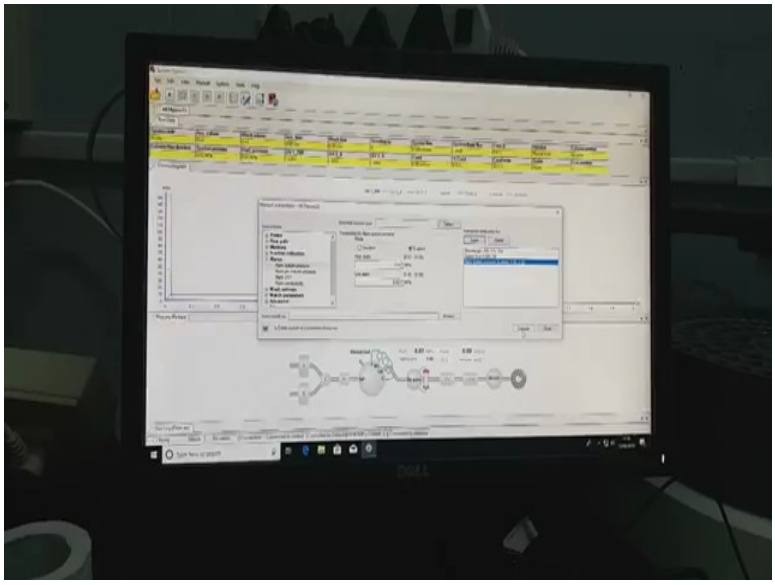

So here the software system flow I am keeping 0.5 ml per minute since we are going to connect the column. So if the flow rate is more than the pressure may come. So after that we have to set the monitors so this is also I am going to set. System flow 0.5 insert and now we have to set alarms at what pressure you need to get alarm. So I am keeping this 0.8 is fine. So once it is done you can insert then execute.

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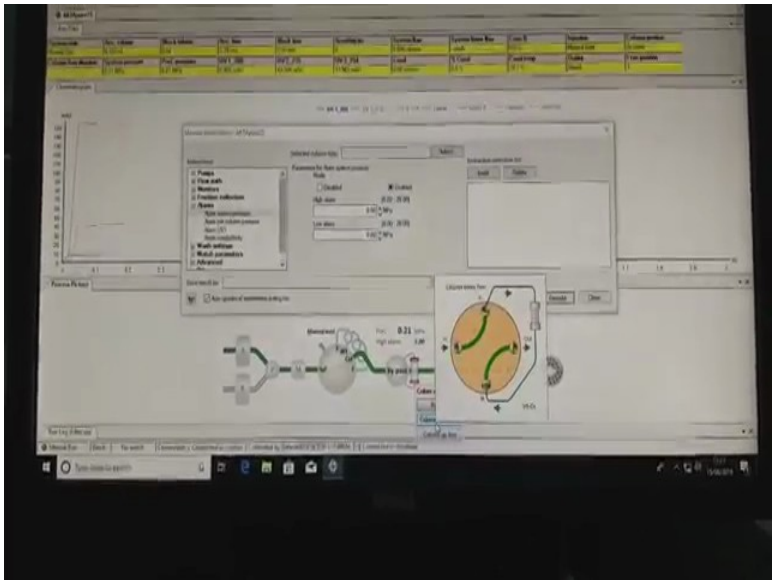

So next here this is the column-connecting portion. So, where her this is the upward portion we have to connect with the column now we are not using column so just we have to go to the column we just click this one column done. So from top to bottom now you can see highlighted one. So you cannot directly connect to the column first you have to fill the buffer or water in this loop. So that there is no air bubbles just open upside of the column with the buffer itself you just directly connects.

After connecting, you have to take out the lower portion of the column otherwise it may burst also but it is not the case because if there is a any high pressure you will get alarm. So here this is the bottom portion of the alarm you can see the buffer here if the buffer is passing through the column so as we can see there is a fill up water in this thing. So once you see complete fill up of this loop or this nob you can directly insert the lower portion.

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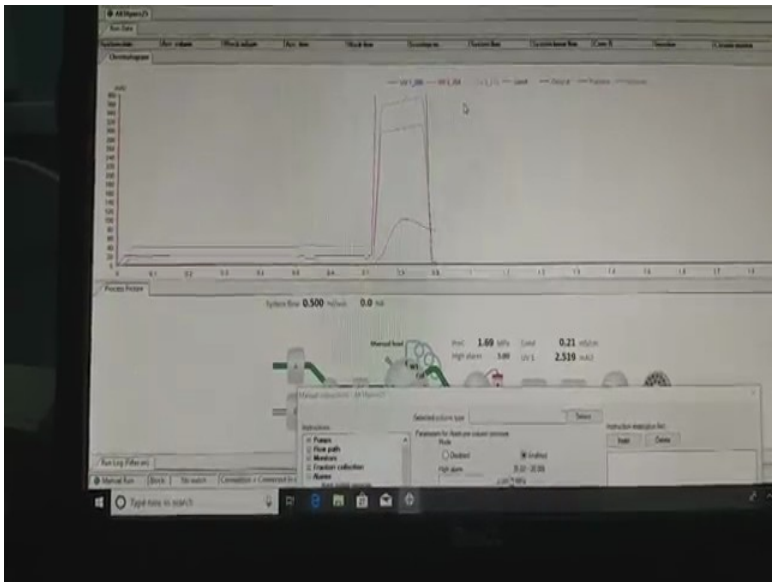

Once you see the buffer filling in we just have to connect with the downward portion. So now the column is connected to the system and you did not touch anything everything will be operated on the software. So here see once the column is connected you can see there is a change in the different UV and conduction of the buffer this is, the red one is the sorry this grey one is the per conduction and that green one is the concentration of P and this are 3 different UV.

Now we are washing with the water so after once completely removed 20 percent (())(20:58) then we will incrementing the buffer. So that the main purpose of the inclination is suppose if you are prepared to your protein solution in a suppose say phosphate buffer. So you have water you are not included with the phosphate buffer then you cannot expect good the

resolution of the goods separation of the protein and also the protein may not be stable in the other condition like in water.

So they may degrade or they may not be useful if you are interested in the (())(26:36) matter reaction. So that is why we always look equilibrate with the equilibrate the column with the same buffer which protein of interest is design. So this will also helps good resolution and keep the intact of the structure of the protein. So we are indirectly we are providing singular conditions for the protein.

So it will behave in a breaking condition. So we completely washed out the internal whatever present inside the column now we will equilibrate the phosphate buffer.

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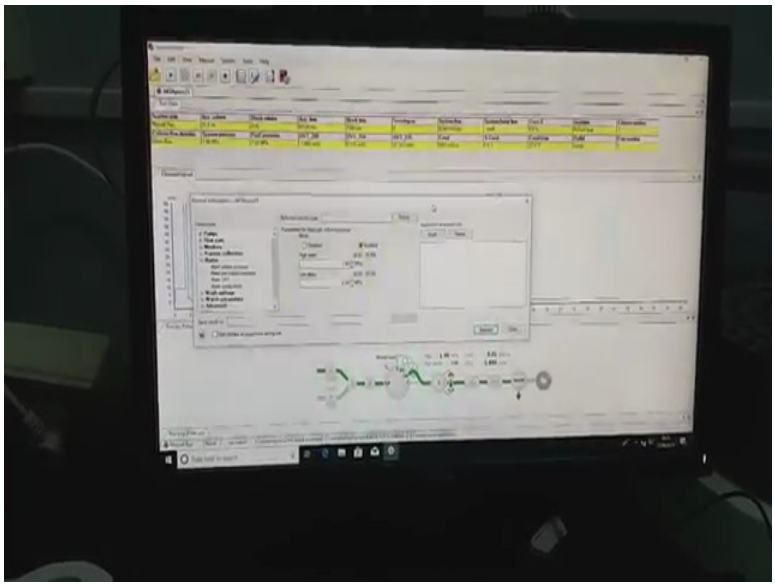

So as we can see here the conductance is completely comes to 0 and we can see there flat line completely flat line flat signal corresponding to UV 280 so that means there is no (())(27:39) inside the column. In addition to that we already washed 30ml of water so the total column volume inside the column is it is around 25ml. So we are so wash 5ml extra. So we can be assure that it is completely removed.

Now what we have to do is we have to just pass the piece whatever we set already without disturbing anything.

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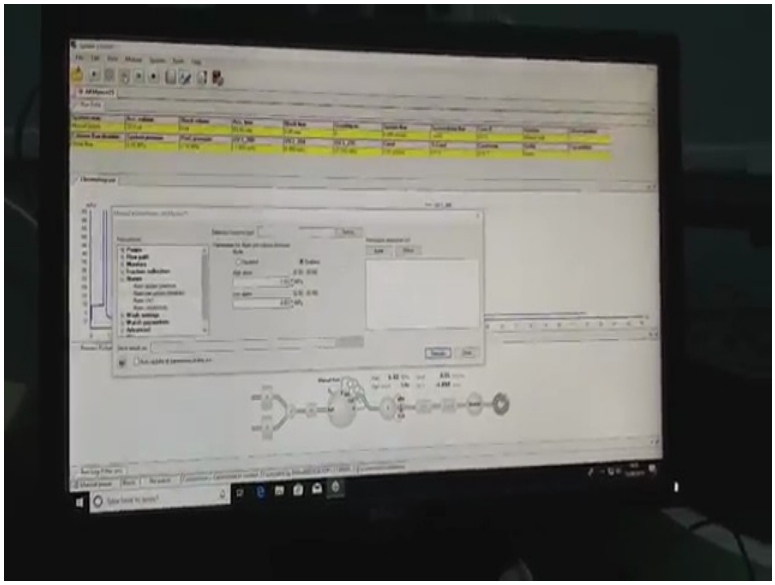

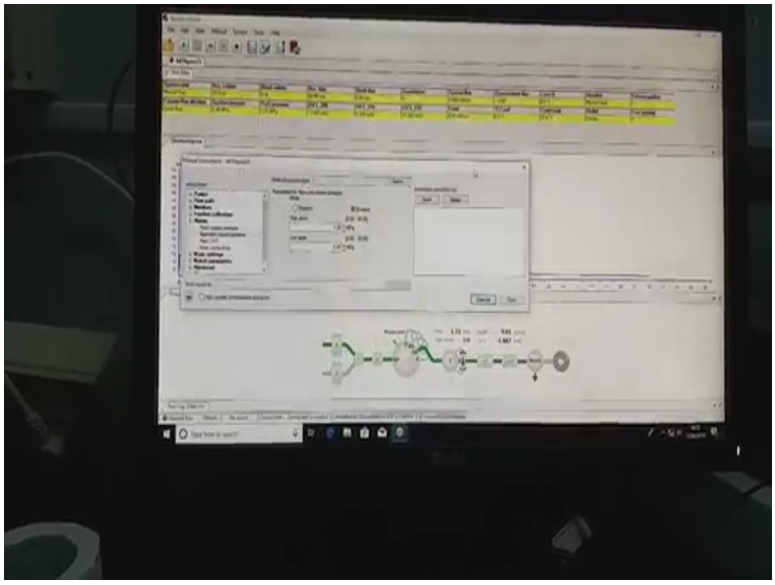

So here we can see a (())(28:13) you just pass then come here and change the into buffer. So once that is done we will reset these things to continue mode. So we can see it is again activated. So we have equilibrated the column using the equilibration buffer. So as we can see here there is a stable line corresponding to UV 280 and there is no other emission can count. So with this we can confirm that we equilibrated the column practically.

So it is time to inject the protein solution (())(29:25).

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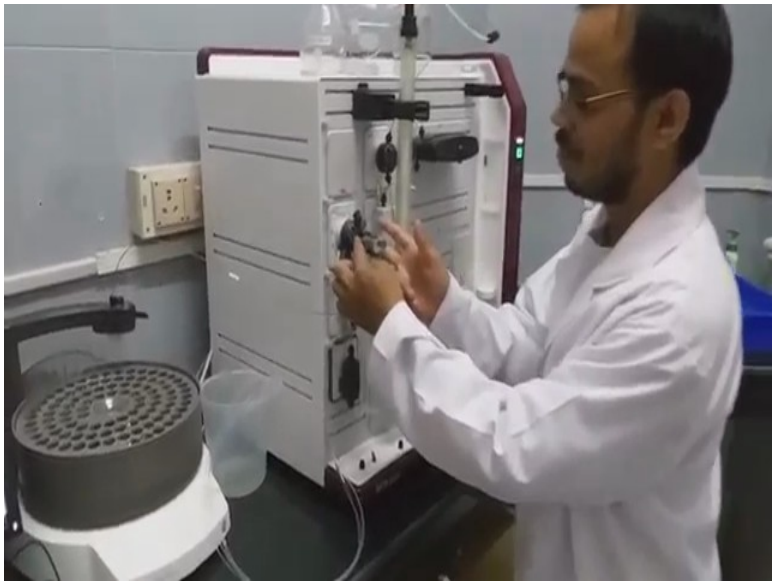

So for injection purpose this is the port where we are going to inject the solution protein solution and this is the lock. Whatever we inject through this injection (())(29:42) it will be stored inside this lock. The size of the lock depends on how much protein you want to inject and the column capacity. So we have 13 reduction column also it can be used up to we can inject up to 1mm.

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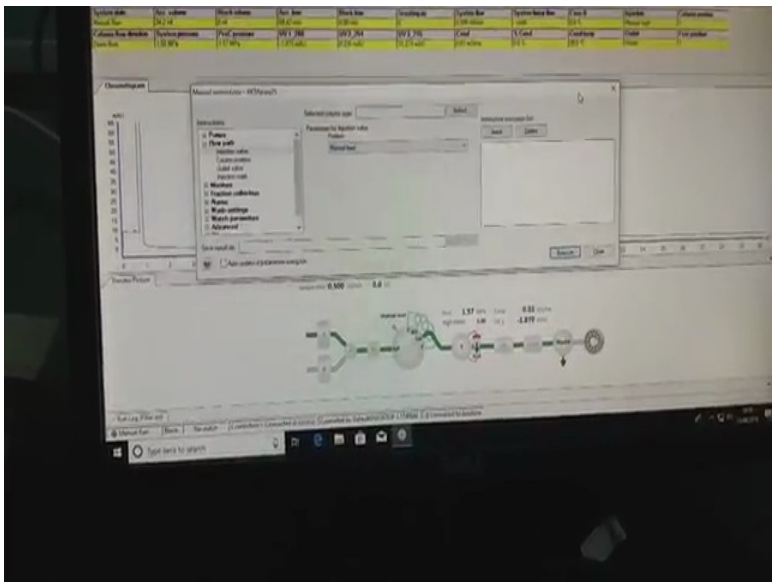

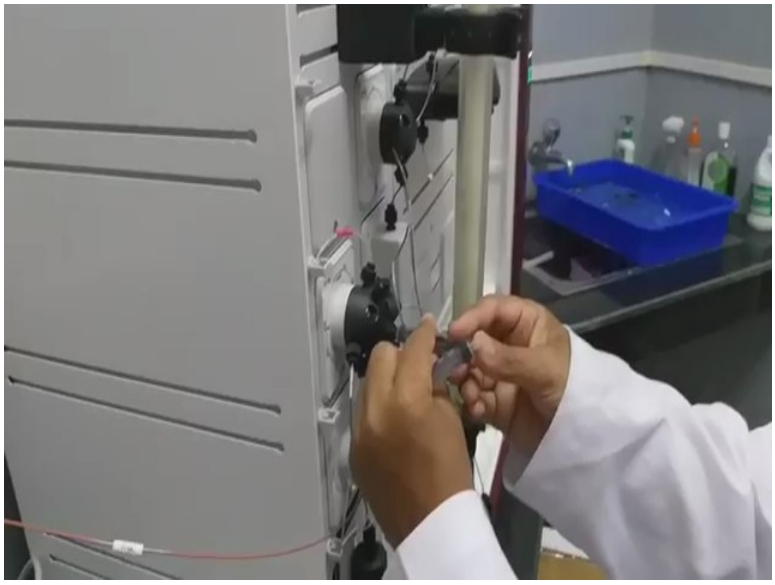

So in this column we cannot inject that much if you want to inject we have the compete different matters with you this kind of loops. So this we will connect as shown in here and we will use for the injecting the protein solution. So what we will do is we have to set few parameters here, so here flow path. So injection 1 you have to show it inject here. So inside this one. Once that is over we just have reset the protein solution and execute the command.

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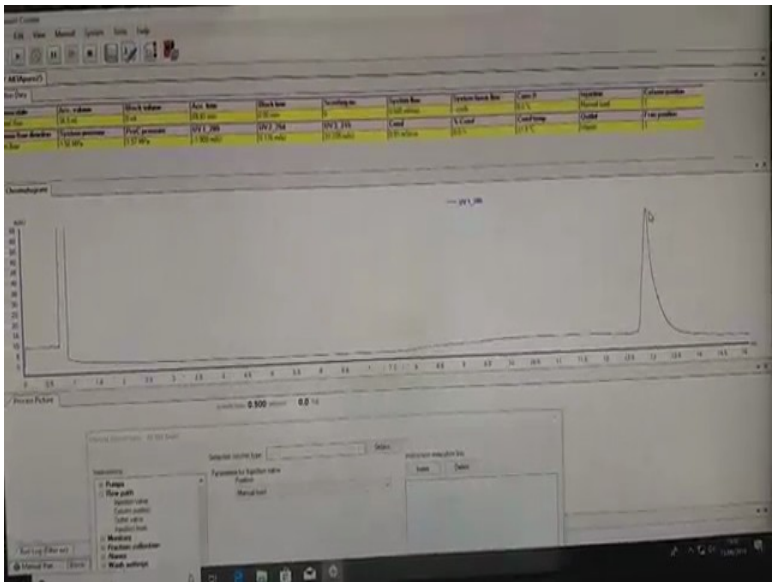

So as we can see here the chromatogram here the protein is emitted so at 30ml of redemption volume. So if you want to say if you want to identify the protein molecular vetarian determine the unknown the protein molecular weight you have to run this kind of analyses like you need to be known that what is the protein molecular weight. So which is available actually commercially available.

So you have to take that protein and just inject based on that you have to construct the calibration curve between log molecular weight and the KV that is partial coefficient which is calculated based on the elution volume subtracted to wide volume divided by total volume subtracted with the wide volume that will give partition coefficient.

So whatever you will get you will get a graph straight line and based on the straight line you can get unknown protein molecular weight. So that can be discussed in size exclusion chromatography.

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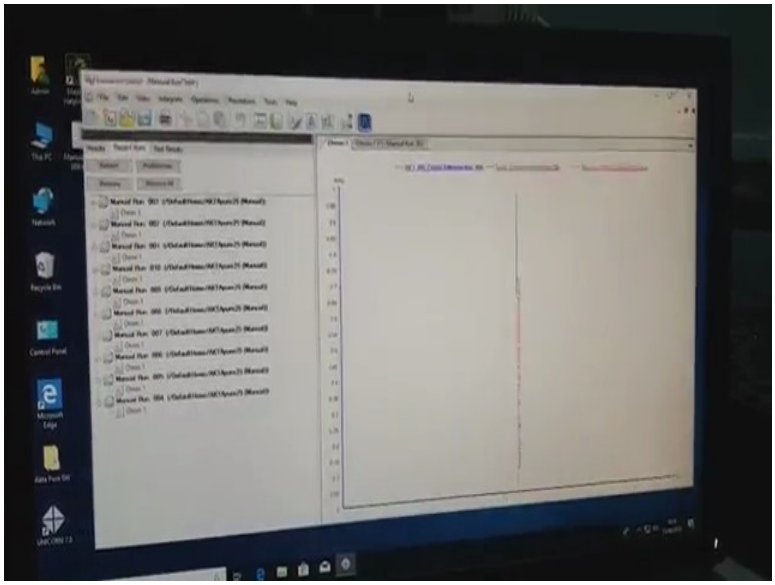

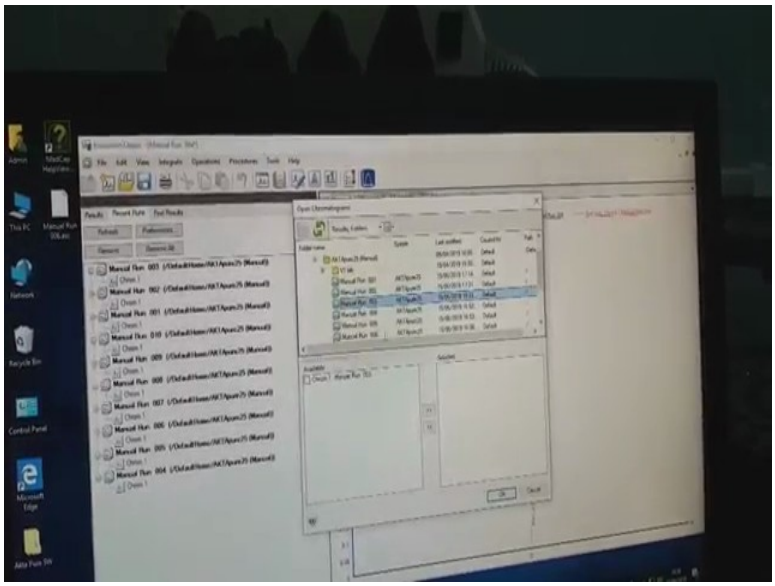

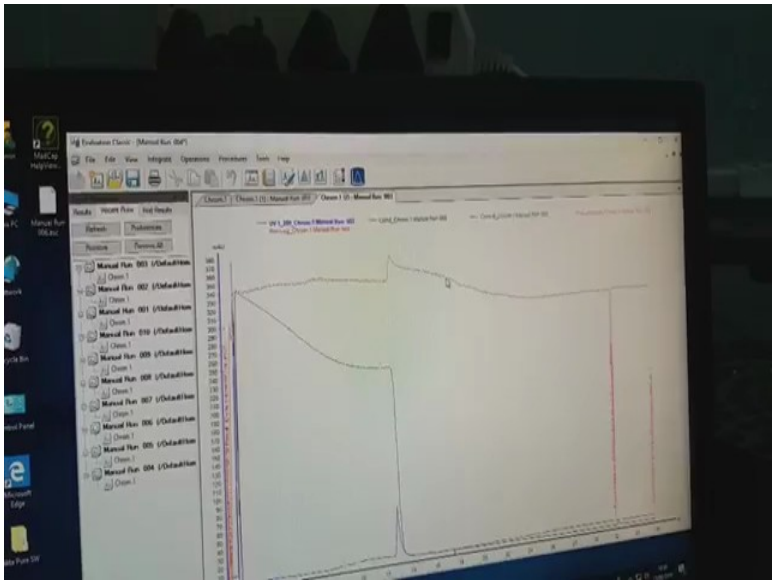

Now we will show you how to analyse the results. So this is the software use it for evaluation purpose evaluation classing. So you have to go to file open chromatograms. So you have to locate where your file is kept just open that one and say ok. So here your seeing so many things, this one correspond into pressure and conductance. So you can customize the things like what you want to see in the chromatogram is only UV 280so you just keep those things and remove all those things ok and here also it is showing 380, you do not need 380 you want to take up to 65.

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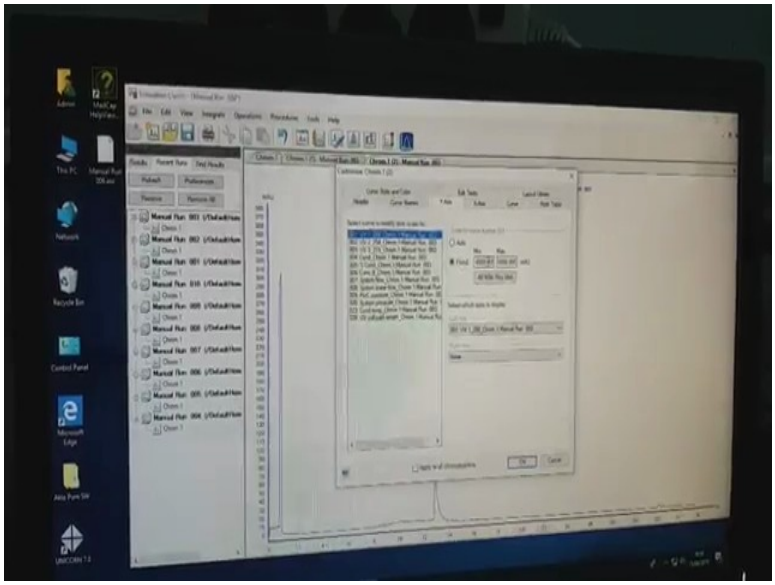

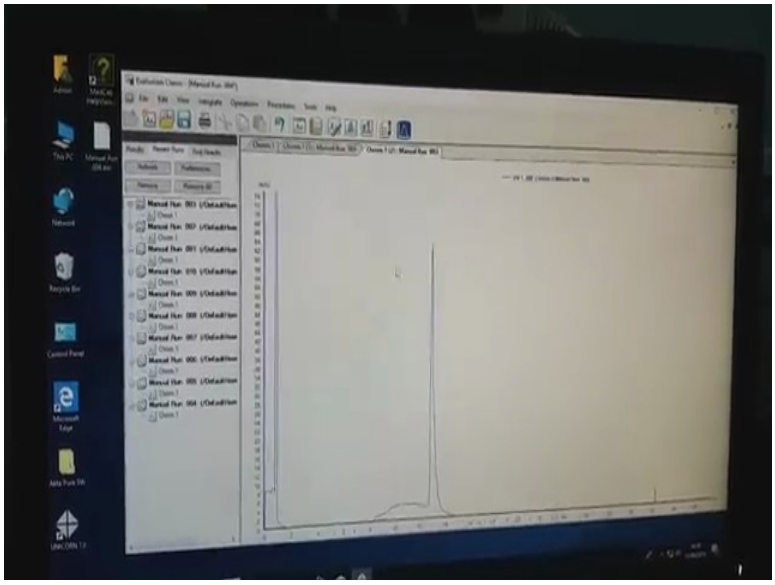

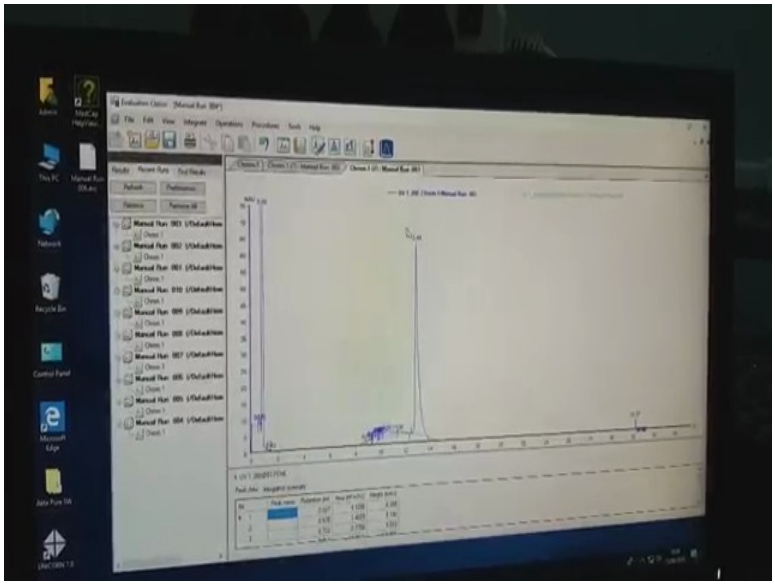

So you just go and change the Y-axis. So you can see this is the chromatogram peak is very sharp. So you can also integrate the peaks so this is UV 280 integration. So just say, so it will give the exact detention volume of the each and every peak how many peaks are horizontal. So here we can see 12.86 this is the major peak what we have. So with this you can analyse the results. So if you want to calibrate you have to calibrate redemption volume for all the protein whichever you are using for calibration and construct the calibration curve between partition coefficient and log molecular weight.

So with this you can identify unknown protein molecular weight. So in this particular FPLC demo we showed you how the instrument works what are the different parts and what are the precautions we need to take while running the instrument and how the software works and how to analyse these things.

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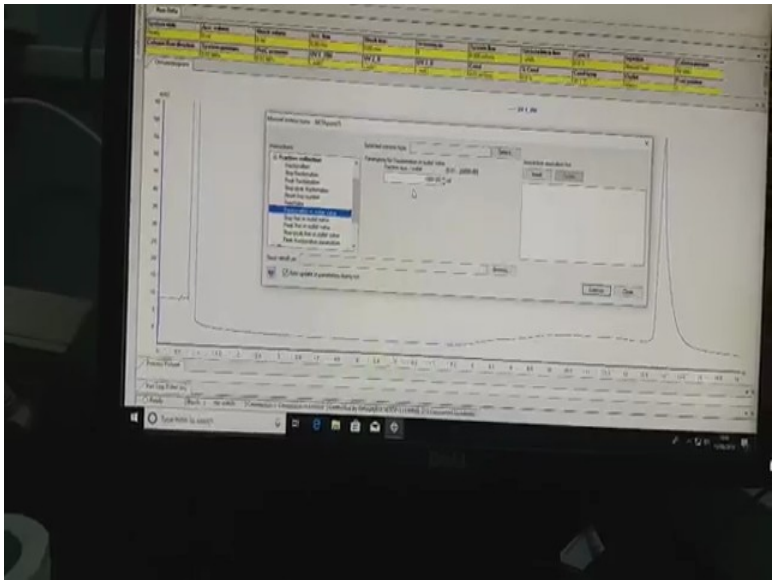

So one other point we forget actually this is the fraction connector. So while your protein is empty suppose your protein is emptying at 12 so started at 12 you want to collect fractions from that time onwards till the end of the emission. So what you can do you can use fraction collector also from the main window this is the fraction collector it will automatically moves. So here contains the censor but you need not to touch anything.

So in main system control here fraction collector there is a option for fraction collector how many fraction you will need to be done when you need to stop fractionation and how much feed to fractionation outlet 1 all these things you can set there in addition to that you can also set system gradient flow suppose you want to elute a protein with the gradient you do not know at what particular concentration in the zone if you are using a nickel nta column or at what particular concentration the protein higher or lower molecular weight elutes.

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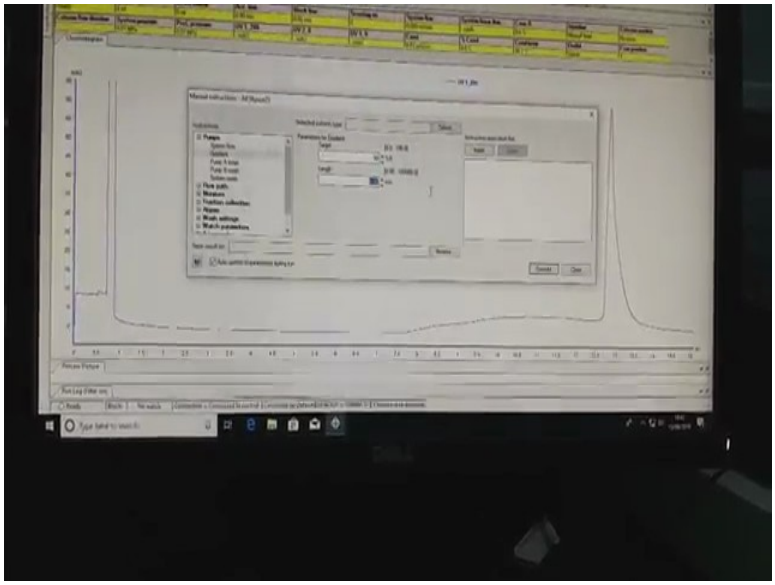

So with this you can just adjust the concentration and length you have to given a suppose 60 minutes. So what system will do it will over 60 minutes of time. It will increase 0 to 50 percentage. So you can do this one reverse ingredient also. So first you will give 15 and time you can keep just suppose 1 minute. So from time when you start the system it will starts with the 50 percent of the peak and reduces to 0.

So all these things will make you familiar how that system works with the first protein liquid chromatography. So hope these things will help you to achieve your goals in your research thanks for watching. Now we understand how a first protein liquid chromatography works. So after using the instrument we have to from buffer to water we have to change the vats because if you are directly keeping in the 20 percent ethanol it may the ethanol whichever present in the buffer and the protein for salts presents in the column they may get restated and lock the column.

So it is a better practice first you change this buffer system to water then wash the rolling whatever the (())(38:33) present inside the column it got eluted then again you change it to the 20 percent ethanol for preserving purpose. That need to remember for better performance of the system.

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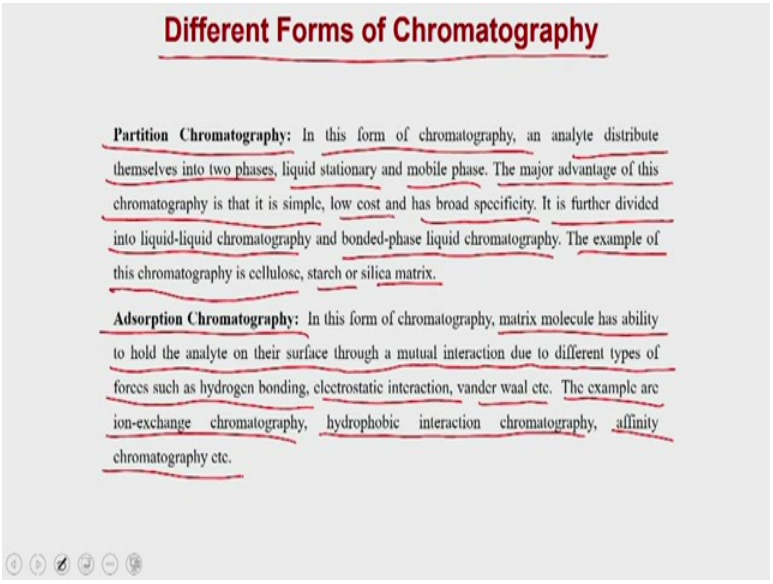

Now the chromatography can be performed in 2 different ways the petition chromatography or the adsorption chromatography. In the petition chromatography the analyse distribute themselves into 2 phases liquid stationery phase and the mobile phase the major advantage of this chromatography is that, it is simple low cost and had a broad specificity it is further divided into liquid-liquid chromatography or bonded phase liquid chromatography.

The examples of this petition chromatography is lowest starch or silica matrix where as in the case of adsorption chromatography the matrix molecule have ability to hold analyte on their surface to a mutual interaction due to the different types of forces such as hydrogen bonding electrostatic interaction Vander waal etc. and the examples are ion-exchange chromatography hydrophobic interaction chromatography affinity chromatography and so on.

So the difference between the partition chromatography versus the adsorption chromatography is that in the partition chromatography the molecule is going to distribute into the 2 phases but the molecule is not going to adsorb whereas in the case of adsorption chromatography the molecule is going to be Partition between the 2 phases one of the phase will be the matrix phase where the molecule is going to adsorb through the non-bonded interaction.

For example the hydrogen bonding electrostatic interaction or Vander waal forces. So with this we would like to conclude our lecture here and in our subsequent lecture we are going to discuss about the difference type of chromatography technique and their usage in terms of the purifying the proteins or protein molecule thank you.