Experimental Biotechnology
Prof. Dr. Vishal Trivedi
Department of Biosciences and Bioengineering
Indian Institute of Technology, Guwahati

Module No # 04
Lecture No # 16
Basics of Chromatography (Part – 2)

(Video Starts: 00:24)
(Video Ends: 01:00)

Hello everyone this is Doctor Vishal Trivedi from department of biosciences and bioengineering IIT Guwahati. And in the demo video where the Banesh is going to discuss about the different aspect related to the chromatography system. In the demo what we have chosen? We have chosen the act up your M as your chromatography system. And we have discussed different aspect of the chromatography system in terms of the operation of the machine and as well as how you are getting the different output in to the computer and how you can collect the fractions.

In addition the Banesh also discussed about the different precaution what you have to take while you are performing the chromatography utilizing the act up your M and I hope you will enjoy the demo and you will understand the different aspects related to the chromatography system.

(Video Starts: 02:02)

In this video we show you how to operate a FPLC Instrument and the basic principle laying mechanism of how separation of the proteins using FPLC. FPLC nothing but fast protein liquid chromatography we can say it is a derived version of HPLC the main difference between HPLC and FPLC is? FPLC can only be used for the separation of the proteins and sometimes small molecules also if we have columns available.

But in HPLC we can use columns for separation of small molecules suppose if you have (()) (02:51) that also can be separated using HPLC. The columns what we will use for HPLC and FPLC also differs. In case of FPLC we will use plastic columns but in case of HPLC columns we will use steel columns. Because the main reason when we are using like this if you use stainless steel columns in FPLC because of the salts, and I concentration of salts and different materials we are using it may corrode this steel.

So that is why there may be improper separation of them component which we want to separate. So that is why we will use only plastic column in this one. The separation of the proteins in through FPLC is base from the size and the shape of the proteins. So if you have like in gel filtration chromatography or size exclusion chromatography in all those mechanism like size exclusion, gel filtration can be applicable in FPLC also.

So it depends on what column you are using. So if you want to purify histamine proteins you can use nickel NTA column (()) (04:27) column. Suppose if you want to only separate hydro low molecular weight protein you can use any gel filtration column suitable for your protein. But the principle behind the separation is same. So these details of like size exclusion or gel filtration we are shown in previous video. In this video we will show how this instrument can be operated.

We are in our lab we are using (()) (04:56) this is from GE health sciences. So all the component whatever we show it is similar in other instruments other company instruments also. But only the architecture of the instrument changes so let see what are the part in contains. So this is the instrument it is connect to a system for observation purpose. So in this system it contains this stationary phase this is the column this is the mobile phase that means a buffers this is the area we will keep all the buffers.

So it starts with the pumps actually. So these 2 are pumps this 1 pump and 2 pump this 2 sets. Whatever buffers are coming from this buffer tray it will enter here ok. These pumps whatever the pressure there they are getting that will be invaded here this is the pressure monitor this. So once here the pressure is monitored it will go to mixer. This is the mixer where 2 different purpose.

Suppose if you are using if you are purifying through nickel NTA column, so in that case you will imidazole in a sperate buffer prepared and one is the equilibration buffer. In that case if you want to elute that protein particular histamine protein you have to mix both the buffer A and B for instance. So those buffers can be mixed here. Once mixing is done it will directly goes to the inlet flow.

Once it entered into inlet flow it will go to this chamber where 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 where the eluted component will be detected. So starting the instrument there is a power button right side of the instrument.

You have to just turn on that instrument then you can see the white light is blinking here that means the system is just started. So after that we will go to this software part. For analysis of any elusion we can use this it come with the instrument it is the unicorn software. We use for analysis purpose so just double click on that one and it will take you to the software. So it will give you 3 popup windows one is method editor, another one is system control and evaluation classic.

So this is system editor where you can see chromatograms and the other one is evaluation classic where you can analyze your chromatogram. Just go for system control the first thing we have to do is connect the instrument. So here you can see the connected instrument AKTApure 25 so just say ok. Now it will connect the system. So this will give different we can change it different commands using this software just go to manual execute manual.

So this is the manual instruction software or dialog box where you can change things. So here different parameters you can change through this pop up window like pumps flow path, and various parameters such as monitor. Next go to pumps here you can change the system flow. So we can keep up to 20 ml if there is no column connected. So normal condition we can keep 5 ml also.

So you just say insert this thing in order to executed by system. Now here it is monitors very important thing we have three wavelengths here we can monitor at 3 different wavelengths so your choice you can give so we are giving 280 to 215 to 254 just insert and say execute. So it started you can see the green path is highlighted and also chromatogram appearing in the chromatogram area.

So it will give 3 different chromatograms so one corresponding to blue that is 280 nanometer for tryptophan tyrosine fluorescence second one is 254 nanometer for a RNA or DNA related and

third one is 215 for petite. So here we can see the path of the flow how it is connected starting from buffer A so here buffer A it will go through the pump and mixing through mixer it will go to all the way to waste.

So here different parameters we can change during running we can change B also. If you want to change B you just say start pump B. So C we can C highlighted area. If you stop the program it will save automatically C you can see some dialog box appears preparing for new run. This is software introduction. These are the buffers we are going to use for this demo. The buffers need to be filtered through 0.2 micron filter and also degassed.

For a degassing purpose we will use (()) (12:21) so it will remove any air any air or a bubbles present in a buffers it will remove those things. So we are using a most common buffer that is a phosphate buffer having PH 7.4 and this is the (()) (12:40) water and this is 20% ethanol. All the buffers were filtered through point 2 microns filter paper and also degassed. So we have watched already the system already been watched.

So now what we will do we will connect to the column. So here precautions need to be taken while connecting these things. So if you are if you have any air bubbles through this loops or the piping system it will directly end up in the column which will destroy it. So to prevent that you have to make sure all the loops and pumps got washed thoroughly and then moving connect in running condition.

Before connecting the column you have to remember few points. This column whatever the beads are there this is in 20% 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 distance between the column filter and the beads the scheduled beads may increase. So that will reduce the efficiency of the column.

So what we will do we have change this pumps into water so we changed into water now we can connect it to the column. Here also some of the precaution needs to be taken. If you are reducing chilled buffer ok suppose you did cold buffer, so that means you have to bring those all the components of the system to the temperature which you want to use for your purification.

Otherwise if you having chilled buffer which directly enter into column that may clog or precipitate 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 connecting the column we need to adjust few parameters. 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 alarm 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 you get alarm.

So I am keeping this 0.8 is fine. So once it is done you can insert then execute. So next here this is the column connecting portion so where here this is the upper portion where we have to connect with the column. Now we are not using column so just we have to go to the column just click this one column down flow. So from top to bottom now you can see the highlighted one. So you cannot directly connect the column first you have to fill the buffer or the water in this loop so that there is no air bubbles.

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

Once you see the buffer filling in you just have to connect with a downward portion. So now the column is connected to the system and you need not to 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's and conduction of the buffer. This is a the red one is the sorry the grey one is the per conduction and the green one is the concentration of the and these are 3 different units.

Now we are washing with the water so after once completely we removed 20% ethanol then we will equilibrating the buffer. So the main purpose of the equilibration is suppose if you are prepared your protein solution in a suppose say phosphate buffer, so you have water you are not

equilibrated with the phosphate buffer. Then you cannot expect good resolution or good separation of the proteins.

And also the proteins 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 enzymatic reactions. So that is why we always to equilibrate the column in the same buffer which are proteins of interest will dissolve. So this will also helps good resolution and keep the; interact of the structure of the protein. So we are indirectly we are providing similar conditions for the protein. So it will behave in native condition.

So we completely watch the (() (20:40) whatever presented inside the column, now we will be equilibrating the phosphate buffer. So as we can see here the conductance is completely comes to 0. And we can see there completely flat line flat signal corresponding to UV 280. So that means there is no ethanol inside the column. In additional to that we have already washed 30 ml of water so that the total column inside the column is it is around to 25 mm. So we also washed with fire mill extract. So we can ensure that it is completely removed.

Now what we have to do is? We have to just pass these things whatever we set already without disturbing anything. So here we can see a pause symbol you can just pause then come here and change the these things to buffer. So once that is done we will reset this setting to continue mode. So we can see this 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 elution coming out.

So with this we can confirm that we equilibrated the column properly. So it is done to inject to the protein solution and (()) (22:53). So for injection purpose this is the port where we are going to inject to the solution protein solution and this is the loop. Whatever we inject through this injection it we will be stored inside this flow. The size of the loop depends up on how much protein you want to inject and column capacity. So we have 1300 HR column also which is it can it can be used up to you can inject up to 1 ml. So in this column you cannot inject that much.

If you want to inject we have the company people might have give this kind of loops. So this we will connect slowly here and we will use for injecting the protein solution. So what we will do is

we have to set few parameters so here flow path so injection one you have to choose inject here instead this one okay. So once that is over you have to just insert the protein solution and execute the command. So as we can see here the chromatogram here the protein is entered so at 13 ml of redemption volume.

So if you want to say if you want to identify the protein molecular the entire determinant in the unknow protein molecules bit you have to run this kind of analysis. Like you need to be known that what is the protein molecular known proteins molecular weight, so which is available actually by commercially available. So you have to take the protein and just inject based on that you have to construct the calibration cut between log molecular weight.

And the kav weight that is participation coefficient which is calculated based on the elution volume subtracted with 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 proteins molecular weight. So that can be discussed in the size exclusion chromatography video.

Now we will show you how to analyze the results. So this is the software used for evaluation process evaluation classic. 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 you can customize the things like what you want to see in the chromatogram is only UV 280. So you just keep those things and remove all those things ok.

So here also it is showing 380 you do not need 380 you want to take up to 65. So you want to go and change the Y axis so you can see this is the chromatogram peak is very short. So you can also integrate the peaks so this is UV 280 integration so just say so it will give the exact retention volume of the each and every peak how many peaks are present. So here you can see 12.86 this is the major peak what we have so with this you can analyze the results.

So if you want to calibrate you have to calculate the retention volume for all the proteins, which ever you are using for calibration and construct the calibration curve between partition co-efficient and log molecular weight. So with this you can identify unknown proteins molecular weight. So in this particular FPLC demo showed you how the instrument works what are the

different parts and what are the precaution we need to take while running the instrument? And how does the software works and how to analyze these things? So another point we forgot actually this is the fraction collector.

So while your protein is empty suppose your protein is emptying at 12 started at 12 you want to collect fractions from that time onwards till the end of the elution. 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 it contains the sensors but you may not need to touch anything ok. So in a main system control here fraction collector there is a option for fraction collector.

How many fractionization you need to be done when you need to stop fractorization and how much feed to fractionization outlet one 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 at what particular concentration of (()) (30:12) if you are using nickel NDA column or at what particular concentration a protein higher or lower molecular weight elutes.

So with this you can just adjust the concentration and length you have to give in suppose 60 minutes. So what system will do it will over 60 minutes of time it will increase 0 to 60%. So you can do this one reverse gradient also first you will give 15 and time you can keep just suppose 1 minute when you starts the system it will starts with the 50% of the B and reduces to 0. So, all these things will make you familiar as how the system works with the fast protein liquid chromatograph.

So hope these things will help you to achieve your goal with your research. Thanks for watching. Now we understand how a fast protein liquid chromatography works. So after using the instrument we have to from buffer to water we have to change the (()) (31:36) because if you are directly keeping the 20% ethanol it may the ethanol which may present in the buffer and the protein or the salts present in the column they may get precipitated and cloak the column.

So it is a better practice first you change this buffer system to water then wash thoroughly what ever is the salts present inside the column it will got eroted then again you change to the 20% ethanol for preserving purpose. That needs to be remember; for a better performance of the system.

(Video Ends: 32:17)

I hope you have enjoyed looking at the demo and you have understood the different operational steps what is required to perform the chromatography. You can be able to modulate these steps because it is possible that you may not have act up M in your laboratory you might have other chromatography system so what I suggest is that you can actually go through with the manual. And the overall the steps are going to be remains same except that the positioning of the buttons and the minute details will might be different. So with this I would like to conclude our lecture here thank you.