Experimental Biochemistry
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Lecture – 25
Protein Purification by Size Exclusion Chromatography (SEC)

Welcome back, I am Aditya Jyoti Basak. And today I am going to be demonstrating to you the usage and working of an F-PLC system. So, basically an F-PLC system is a fast protein liquid chromatography system. It is a machine which helps in purification of proteins. So, what we have here is a semi automated machine which makes life easy; when you mix separating a mixture of proteins.

So, what I have in front of me is an F-PLC system from a certain company. So, these machines will vary slightly based on the company they are purchased from or the model that they have so different labs have different models. And there will be some slight variations according to that, but in general what am going to talk about today will cover the basic principles which are going to be present across all F-PLC systems in general.

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So, today am going to be demonstrating an experiment which is size exclusion chromatography experiment. So, basically what I am going to do is am going to use a size exclusion chromatography column that you can see we have here and use it to separate out proteins based on their overall molecular size and shape. So, in very simple

terms if I have a mixture of three proteins having three different sizes; of say example, 150 kilo Daltons, 50 kilo Daltons, and 10 kilo Daltons all mixed together. Using size exclusion chromatography I will be able to separate them out into three separate pools.

So, the size exclusion chromatography column that I have here today in front of me is a preparative grade size exclusion chromatography column. So, this is used for separating out mixtures of proteins. What I have here is a 16 to 600 superdex, 200 preparative column. So, basically this can separate out proteins ranging in size from approximately less than 10 kilo Dalton to up to 500 or 600 kilo Daltons. So, basically as I was saying or as you might be knowing; when you extract or isolate proteins from bacterial organisms they will have a large number of proteins.

And size exclusion chromatography column or size exclusion chromatography is a step which will allow you to separate out different proteins and thus purify your protein from other contaminant proteins. The principle on which this works is that larger proteins will have a larger hydrodynamic radius. And they will migrate differently through the gel matrix compared to smaller proteins which are much more globular in shape. So, this size exclusion chromatography column is made up of a matrix as you can see here from here to here this white gel like substance is the matrix of the column.

And we know that it has a volume of 120 ml. So, basically this matrix is composed of beads which have very tiny pores of varying sizes. And if your mixture of proteins has let us say it has there are three different proteins which are present in your mixture. One of let us say; 300 kilo Daltons, one of 100 kilo Daltons and another of 50 kilo Daltons. Once it starts passing through this matrix of the size exclusion chromatography column, the smallest protein that we have 50 kilo Dalton it will be able to travel through all the different pores that represent in the matrix.

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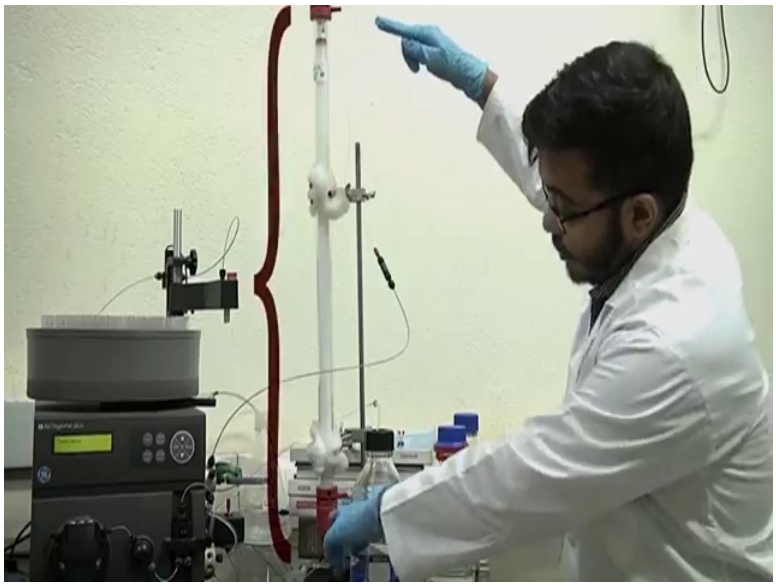

So, it will travel through all the small pores present in the beads and as a result it will actually take a lot of time to pass from this point to this end point of the column. However, the large proteins that are also present as contaminants, they will not be able to enter all the pores. So, some pores which are very small will not physically allow the large proteins to enter.

So, they cannot spend time traveling through those small pores maybe they will travel through the larger pores comparatively larger pores. And as a result they have less path to travel and hence they will elute out earlier than the smallest protein. So, basically once you are passing a mixture of proteins through the top of the column by the time they are passing out of the column the largest protein will come out first followed by intermediate sized proteins.

And the smallest proteins which had much more liberty to travel through all the pores in the matrix will elute out at the very end. And later on I am going to show you how this helps us in separately collecting the largest protein the intermediate size proteins and the smallest proteins and thereby purifying our protein mixture. So, in earlier times before the advent of advanced technology what people used to do is they used to take glass columns this tall or maybe even taller.

And they used to pack it by adding the gel matrix that we have here they used to gradually layer it into the entire glass column. And once it was very nicely uniformly

packed they used to take a certain amount of the protein mixture and gently added to the top of the column. And basically gravity separation is how you pass on throughout the matrix and then elute out at the end. However, nowadays due to the advent of technology we have machine systems here which we refer to as F-PLC.

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And basically what this machine has is one of the key components is this peristaltic pump that is present here. So, basically this pumps the protein from the inject once I load the protein into the machine this pump will drive it through the column and allow me to separate out the proteins.

So, basically if you do it using gravity separation an entire experiment which could last more than a day can get completed in a couple of hours because; of the presence of a pump which makes things faster and makes life easier for us. So, I will just give you a brief overview of what all these wires are. I know it can be a bit daunting for beginners, but it is really simple once you understand what is happening

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So, you will notice that in this bottle I have some liquid. So, basically this liquid is the buffer in which I will purify my mixture of proteins. This buffer inside this you will see there is a tube if you will see this tube and there is a nozzle at the end. I dip the nozzle into the buffer and later on as you will see during the live demonstration. Once this machine is switched on and is working, it will pump out buffer from here.

So, it will take up the buffer through this channel it will take it out through this channel and bring it to this mixer. And from here through this complex network of wires tubes rather, you cannot see it because the tube is now below the machine it comes here. So, this is where the pump is actually taking a buffer from here. It will then travel to these fine through these fine wires into this portion of the machine. So, this portion of the machine has a large number of valves which direct the flow of buffer.

Normally when nothing when we are not purifying proteins, but we have kept it on; it will take a buffer from here via this pump bring it to this point and then it will pump out the buffer into this conical flask that I have here. So, basically this collects the buffer from this vessel into this vessel. So, colloquially I just call this the trash vessel because I keep unwanted or excess buffer here.

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You will notice that there is an injection here. At this point I actually have an option where I can take my protein sample and using this injection load it onto this loop. You see this coiled loop. So, this has a fixed volume of 2 ml and I can take 2 ml of protein sample and inject it into this loop where it stays here.

Once I push a few buttons and change the valve orientation for this machine; what will happen is the buffer that is being pumped here. Instead of going directly to trash, will now travel via these nozzles and push the protein out of this loop and carry it on onwards up to this column. So, basically once I have my protein here and am ready to start the experiment I will push a few buttons. And then instead of taking the buffer from this vessel to the trash vessel it will basically start pumping it through this column.

Keep in mind that this column will also be pre equilibrated which means pre soaked with this buffer before I introduce protein into it. So, this valve setup will allow me to now send proteins mixture of proteins through this column. And the pump will keep on pushing the proteins and along with buffer until the entire column has been traversed by the mixture of proteins.

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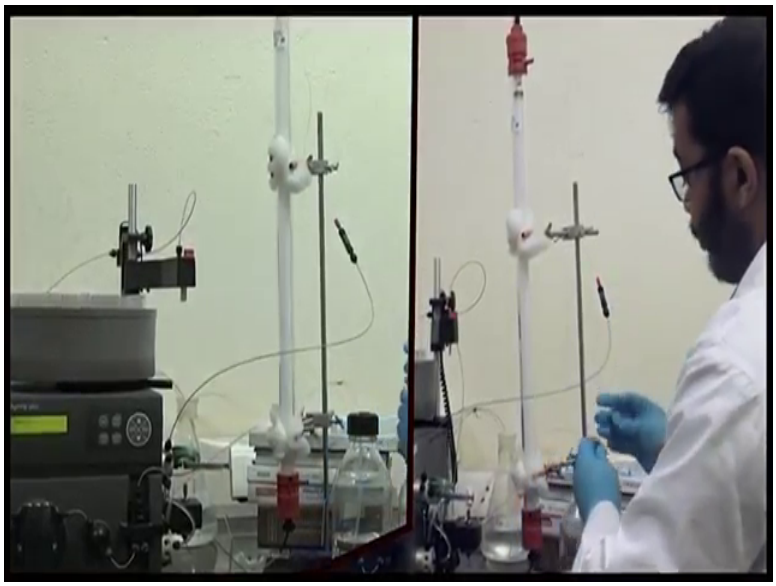

So, during olden days before the advent of modern technology what scientists used to do in case they wanted to do size exclusion chromatography was; they used to take long glass columns as long as this or maybe even longer and then they used to pack those columns with the gel matrix. So, they had a slurry of gel matrix and they used to add small amounts at a time. And after a time period they used to uniformly pack the matrix inside the columns.

Once they have done that they used to take the mixture of proteins and gently add it to the top side top surface of the column matrix and wait for gravity separation to separate out the proteins. So, basically they loaded on the protein and then the gel matrix keep in mind in size exclusion chromatography always has to be soaked in the buffer in which the proteins are present. So, they set up the matrix soaked it completely in the buffer in which proteins are present and then they added the proteins. And then manually they used to keep on adding buffer to the column.

So, each column will have a specific volume and beyond that as you keep on adding buffer on this side buffer will slowly drop by drop come out from the bottom part of the column. And once after you have layered on the protein mixture and you keep on adding buffers. Gradually with time if you keep on collecting the buffer drops you will see that after a long period of time, the entire mixture of proteins has been collected out has eluted out of the column into separate batches.

You can understand how tedious and time consuming it was to add small amounts of buffer continuously at a time in order to prevent the matrix from drying up. And also what they used to do is they used to take small tubes like this small append of tubes and they used to collect fixed volumes or fixed amounts of the elution elute that used to come up. So, suppose they collected 10 drops then the took this tube away immediately they took a new tube and collected another 10 drops and they used to keep on doing this in a coordinated fashion.

So, you keep adding buffer here and you keep adding buffer here. And once the entire volume of the column has been replaced by fresh buffer they know that all the protein mixtures have come out into the eluted tubes. And then they used to test for the presence of protein in those eluted fractions by checking the absorbance at A 2 80. So, you can very well understand how tedious and time consuming and laborious that technique was. However, now due to the advent at due to the advent of technology we have F-PLC systems.

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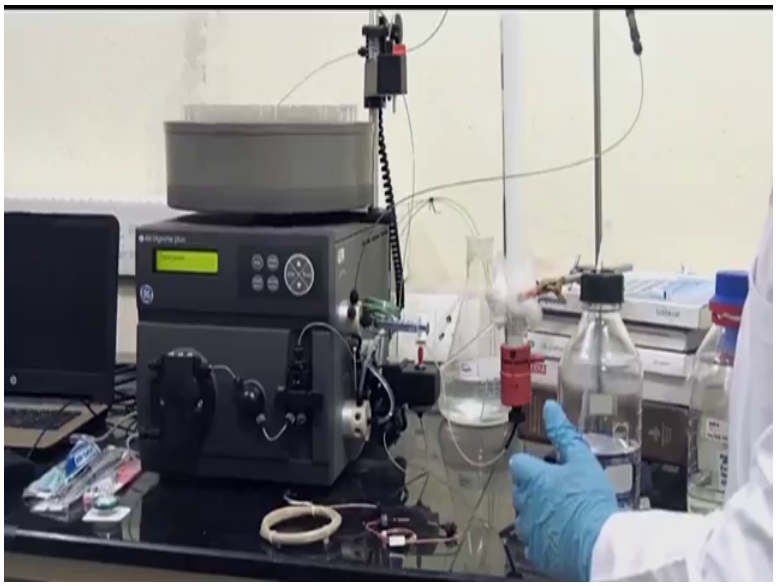

So, there are different F-PLC system some are semi automated or some are fully automated, but in general what they have is they have a peristaltic pump. So, basically the peristaltic pump takes buffer from a container and keeps pumping it at a constant velocity through the column at a constant rate through the column. And once the protein has been added into the column the pump keeps on pushing it down throughout the entire

column. And you can get purified proteins in a matter of a couple of hours. So, it makes life easy and fast for all of us.

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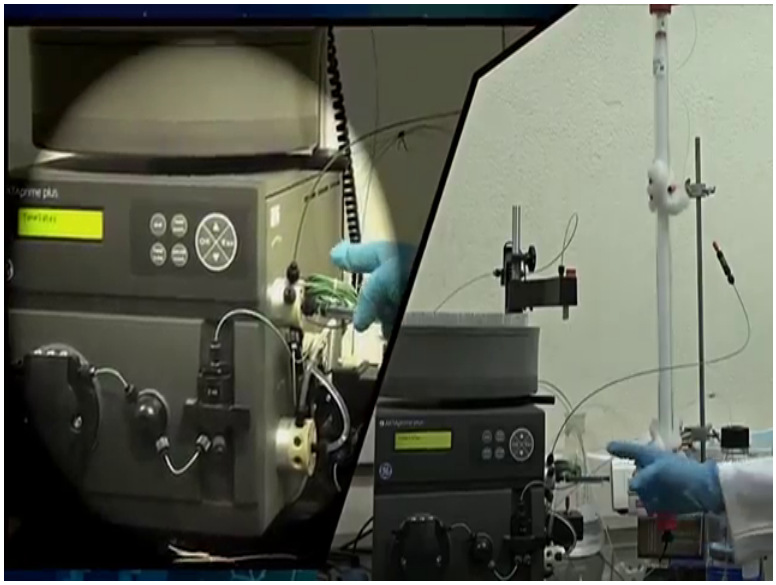

So, if you look at this area of the F-PLC machine so basically this is an injection valve. It has different nozzles we and the communication between the different tubes can be controlled by a few buttons in the machine it is not as complicated as it seems. So, basically what happens is in the default mode buffer is being pumped from here traveling through these tiny tubes very narrow tubes and it is coming here.

And here I can set up different options for the machine in one option if what I can do is the buffer can travel through this flesh coloured tube. And go into the trash vessel as I had shown earlier. Another option is the buffer can instead of traveling here; if I change the mode of the machine the buffer will then travel through this fine tube which you can see here.

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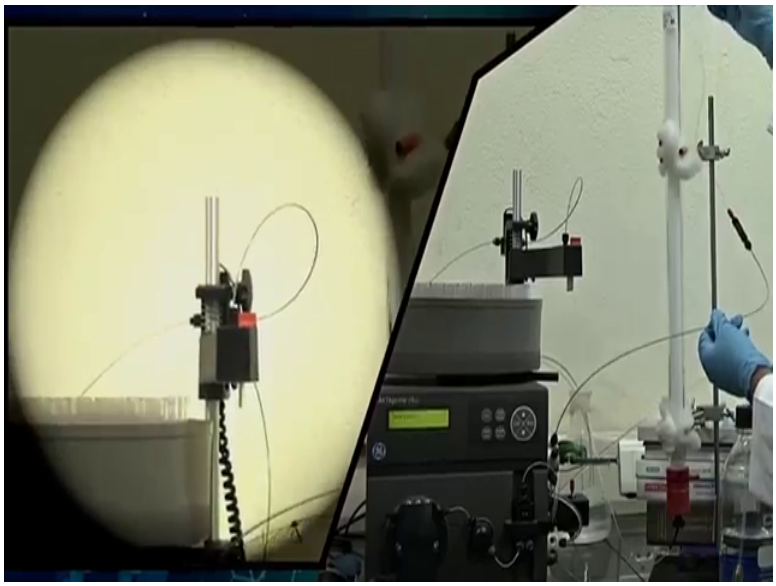

And then through this transparent tubing it will come to the top of the column and it will pass through the column. So, basically once I change the valve position buffer which is coming from this vessel will then travel through this tube and will reach the top of the column and will start passing through the column. After which it will travel through the subsequent tubings and ultimately it is fate is what I will discuss subsequently.

Before we really start separating out the protein mixture I keep on stressing this information that your column should be completely equilibrated or completely soaked with the buffer in which you want to purify your protein. Now this column that I have here today has a volume of 120 ml. So, basically; that means, that using this machine I can set a flow rate of 1 ml per minute and pump this buffer at a rate of 1 ml per minute from here and pass it to this column thereby equilibrating it with the buffer.

How this happens is buffer once I have set the valve in the correct position buffer which is traveling from here will then travel through this tube and reach the top of the column. And at a flow rate of 120 ml per minute it will take 120 minutes for this column to be completely equilibrated with the buffer that I have here today. Now, after only after the column has been equilibrated with the buffer; can you inject protein into the column? So, how do we do that? You must have been noticing that there is an injection connected here.

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So, basically this is a valve through which you can take up your protein sample and inject it into the machine. What happens is, once you have injected if you I will demonstrate this later on in detail. So, basically you take up some protein and then you fit it to that nozzle. And as you keep pressing it you are injecting your protein into this particular loop.

So, you will notice that this injection valve has a large number of nozzles and tubes connected to it. So, this tube is the tube into which you will store your protein mixture which you want to purify. So, once you have injected it here, you can keep it like this for some time. Until and unless your column is completely equilibrated you keep your protein here. Once it is here what you do is, you again press a few buttons here and you change the valve orientation of the machine.

After that what happens is buffer which is already being pumped through these tubes will now instead of directly travelling through this tube to the column, will first travel through this tube displace the protein which is already injected into it into this tube. And then push the protein mixture along with buffer to the top of the column. So, that is how you inject your protein sample onto the top of the column. So, this loop that I have here is a 2 ml loop. So, basically you can load 2 ml of protein mixture using this loop.

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There are other loops that are present for example; I have here with me a 5 ml loop. So, in case you have a large volume of sample 5 ml then you use this loop instead of this loop. You just detach it from the valve system and then reattach this loop in that place and then you can work with a large volume of sample.

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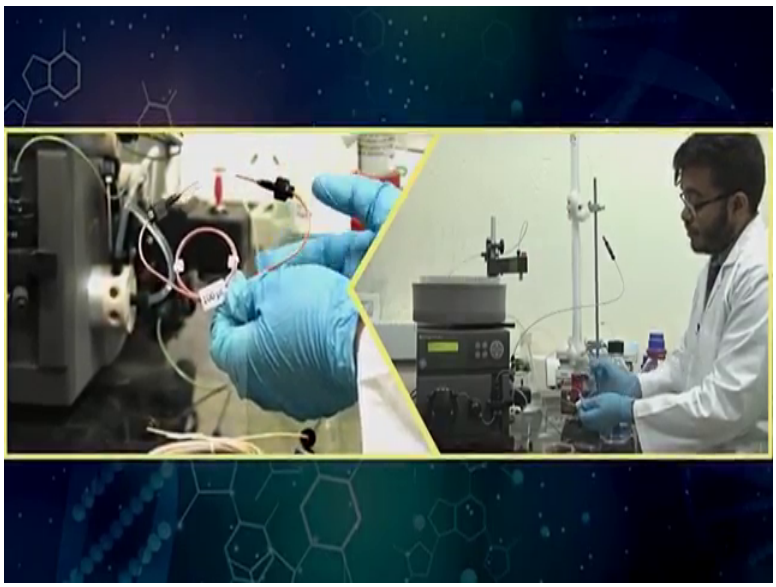

Sometimes when you are working with proteins it is often not possible to have very large amounts of protein. So, in that case or if you need to work with very concentrated protein samples then; we have loops which have a smaller volume. For example, this is 100

micro liter loop it is volume is 100 micro liter inside the tube. So, if you have a small sample of 100 micro liters you do not want to dilute it into 5 ml loop.

You just change the loop here and insert a 100 milliliter loop and then using the same principle you just load it onto the top of the column. So, till now have shown you how we load the buffer into the column and subsequently how we will load proteins into the column. But where do these go?

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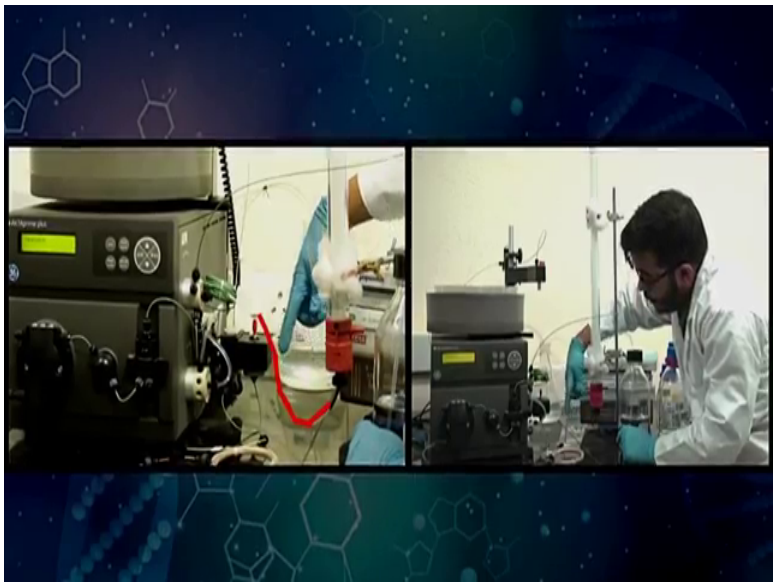

So, you will notice that at the bottom of the column there is this tubing which is protruding out. So, basically as you keep pumping liquid into the top of the column, liquid from the bottom keeps getting displaced out. So, you will notice that the liquid will travel from the bottom of the column via this narrow tubing.

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And then it will reach this portion of the instrument. So, basically this is a UV detector which can check the absorbance of the solution that is passing through it at 280 nanometers. As you know that proteins which have 2 to 5 tyrosine and to some extent as well also phenyl alanine. They all show an absorbance at a 280. So, this is the primary detector that we use to check for the presence of protein.

Once only during the course of the experiment when only buffer will be traveling out from here to this machine it will show very small amount of absorbance at a 280. It is only when proteins start coming out of the column that they will show an increase in the value of a 280. And we will then be able to recognize the portions of the liquid in which proteins are present.

So, the liquid enters here into this UV detector and it comes out from the bottom via this green tube and then it enters this portion of the machine which is basically a new a portion which detects the conductivity of the solution. So, not just the absorbance we can also monitor the change in conductivity of the liquid that is coming out through the column.

So, in certain experiments even this provides important clues as to what is being purified at which stage. After it passes through these detectors what you will probably not be able to see at this angle is there are different options. After passing through these they can directly travel to the trash vessel.

So, in case only buffer is being passed I and I do not want to collect it once it is come out of the column and pass through these detectors they will directly go into this conical flask which collects the trash liquid. However, again using some buttons in this machine instead of sending it to trash I can also send it via this tube into this arm.

So, what happens is once it reaches here it finally, travels through this tube this portion and then it drips out. So, what happens is this portion of the machine that am showing now; so this and this is collectively generically called as the fraction collecting system. So, drops of liquid will fall from here and I will show you we actually have tubes here we have different tubes that are collect present in this fraction collector drum.

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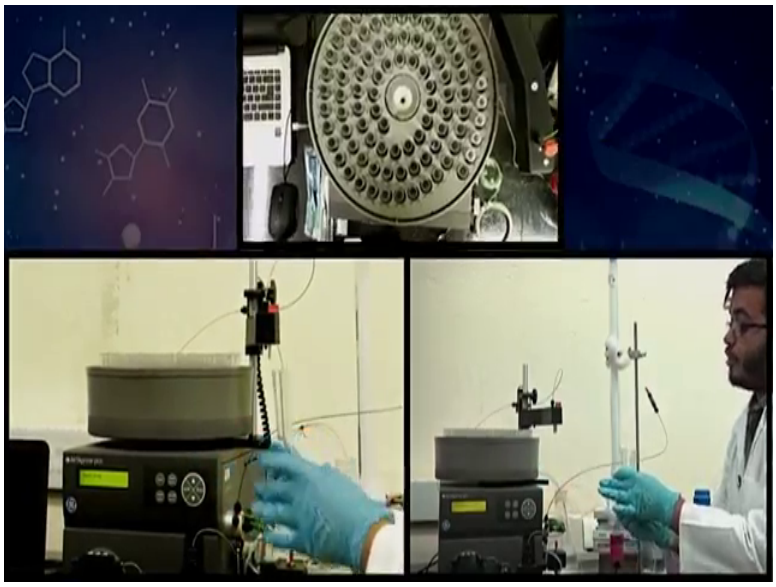

So, you will notice that these tubes have a certain volume. So, what I can set is often say 1 ml of liquid has come out through this tube this will rotate. And the next tube will come in position below this outlet and it will collect the next 1 ml after which this rotates by a certain amount and the next tube fills up. So, although manually I am rotating the drum right now; what actually happens during a real experiment is that it is all completely operated by the machine it is fully automated.

And you do not actually have to wait and collect equal volumes of the liquid that is eluting out. It makes life easy and also later on as I will show you later during the actual experiment all of these tubes are numbered. So, you can actually track that what amount

of solution is collected in tube number 10, or tube number 15, or tube number 20, so on and so forth.

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So, now am going to demonstrate the utility of this instrument by actually carrying out an experiment in which we purify a mixture of proteins that we have here in this wire. So, this mixture of proteins has already been passed through an affinity chromatography column so it is partially purified. However, in spite of that step of purification there are still mixtures of different sized proteins which we are going to resolve using our size exclusion chromatography column.

So, first thing that am going to do is; am going to take it up in this syringe here it is simple, but try to be careful. So, that you can introduce as few bubbles as possible if you end up with two frothy solution or two bubbly a solution it is not good for your protein it is not good for the machine. So, it is best to avoid that as much as possible. So, I have taken up the protein solution into my syringe.

And now what am going to do is I am going to pass this through a syringe filter. So, what I have here is a 0.2 micron syringe filter. So, what am going to do is I am going to attach this to the nozzle of my syringe and the material that this is made of has pores in the order of 0.22 microns. So, it will get rid of any aggregates or insoluble material which is present in this protein solution.

We do this in order to protect our column because in case such insoluble or large sized aggregated material gets introduced into the column. The column that matrix will get clogged up and in future this will damage the column and reduce it is efficiency and even it is lifespan. So, what I am now doing is; am going to attach the syringe filter to this syringe so simple process. And then am just going to pass it through this syringe filter into this new container.

So, this new container now contains my filtered protein solution. So, which is devoid of any large suspended impurities or particles, which could have damaged the size exclusion chromatography column. So, right now what I have with me is filtered protein solution which I am now going to inject into this machine.

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So, now we are actually going to start an experiment using this instrument. So, before you start this machine always make sure that your buffer container is filled up with buffer. Ideally it should be filled up to the brim. But; however, from experience I know that the experiment that am going to do today will not need this much amount of buffer that is why it is a half filled bottle.

But if you are a beginner or if you are not very comfortable it is always better to have excess buffer in your container. Because otherwise in case your buffer gets depleted and the machine is still switched on what will happen is instead of liquid being pumped into

the machine you will introduce large air pockets and that is going to damage your machine completely.

So, always be very careful that you have sufficient quantity of buffer before you begin the experiment and switch on the F-PLC pump. So, now, as I am going to show you I will just navigate through the control panel of this instrument. And once I reach manual run I will set up a manual run I just go ok. And so there are different options like in case; am working with two different buffers buffer a and buffer b and buffer 1 and buffer 2.

In that case I can actually set the mixed percent final mixture of buffers that is going to travel through the column. So, this sort of buffer mixing is required once you are doing affinity chromatography or some other types of chromatography. However, for size exclusion chromatography that we are going to do today it is not at all necessary. Hence the second buffer percentage is set at 0. We have only one buffer so second buffer or buffer b is 0 percentage is required so that is what am setting up here.

Next what I will set up is the flow rate. So, as you can see it is in ml per minute what am going to do is am going to set up the flow rate at 1 ml per minute. So, that is the rate at which buffer will be taken up from the container and will be passing through the entire system tubing. So, I just set up the flow rate at 1 ml per minute. Next what am going to do is pressure. So, keep in mind this is an F-PLC unit.

So, F-PLC systems use glass columns as you as you have already seen the column that we are using is a glass column. And glass columns cannot withstand too high pressure. So, if the pressure goes up beyond 0.5 mega Pascal as am setting it up right now. So, I have set up the pressure limited 0.5 mega Pascal if the pressure difference across the glass column exceeds 0.5 mega Pascal the machine will give an alarm and it will shut down the system.

Because once it goes beyond that you will end up breaking apart your glass column. So, that is very important these glass columns are very expensive. So, always set up the pressure limit at a safe value. So, am going with 0.5 mega Pascal pressure limit. And what am going to do is now that I have checked the buffer value the amount of buffer is and the pressure limit has been safely tuned; am going to start the machine. So, I will press ok, so it is.

Now what is happening is buffer is being transferred. Since it is a colourless liquid you will not be able to see anything, but what am telling you that is happening is happening is buffer is now being pumped from this bottle at a rate of 1 ml per minute and it is traveling through the tubing system. However, I have set up the valve position in such a manner that the buffer is now traveling to waste.

In fact, I can show this to you if you will see this here; I do not know if it is visible, it is probably visible can you see small droplets of water that are falling into the conical flask. So, basically buffer is traveling from here traveling through the entire tube and is traveling here. So, and is getting collected in the trash conical flask.

So, you might ask why I am doing that why am I wasting buffer. The reason is this machine is not running continuously. During experiments it is running after which is being shut down and it is staying like that for extended periods of time before the next experiment. And what can happen is sometimes in these tubes air pockets might develop there might be a gap the column the entire tube might not be continuously filled with liquid there might be an air gap.

And if you do not get rid of that by flushing the entire tube with buffer you risk introducing air bubbles to your column which I keep on repeating is very deleterious for your column. And you will end up damaging your column and reducing it is lifespan and also you will mess up your experiment. So, what am going to do now is, I am going to flush the tubing with this buffer for some time. And meanwhile what I will show you is.

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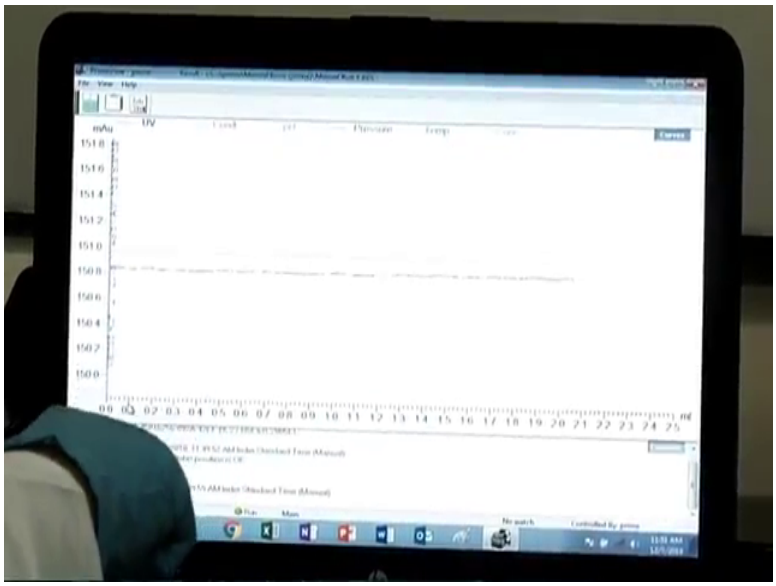

If you can see here this is the computer that is connected to this machine. And what this shows is in the x axis I will set up the base as volume. So, basically it will show how much volume of buffer has passed through the tubing. And on this axis you can change the axis you can show the ultraviolet absorbance at 280 nanometer you can monitor the conductivity, you can monitor pH, you can check the pressure that is developing across the column, you can monitor temperature and concentration.

Since, we are working with protein solutions the best parameter that we need to look at or the easiest parameter that will confirm presence of protein is absorbance at 280. So, I am setting the y axis at 280 nanometers and this is what we are observing right now. If I zoom in right now since nothing is actually traveling through the column it is all a straight line. So, basically it is showing the absorbance and the, if you want you can look at the temperature, or the pressure, or even pH, and conductivity.

So, these are versatile machines which can measure a lot of thing. Right now we are interested only in the absorbance of 280; for the liquid passing through the detector in this machine. So, while am flushing the tubing with this buffer what I need to also manually do is clean this loop. So, if you remember this is the loop into which am going to keep my protein. And once everything else is ready the protein will be then transferred from this loop into this column.

So, before I actually load my protein into this loop I need to clean it up once. And what am going to do is I am going to clean it with this same buffer. So, that protein does not precipitate out inside this loop. So, basically I will fill this loop with this buffer and this buffer will displace the earlier liquid that was present inside it. So, I have here a certain amount of this buffer and I will just take it up in to this syringe.

So, while you are working with syringes be very careful do not discard them either here and there always be very careful. Because these things can lead to accidents which can easily be prevented if you are a bit careful while working. So, as am going to show you right now. I have taken up a volume of around 4 to 5 ml of around 5 ml of buffer. And now what am going to do is I will just get rid of a few bubbles and this is a 2 ml loop.

So, this is a 2 ml loop and I have 5 ml of buffer. So, once I fit this syringe into this nozzle and I press it here, what will happen is; I have not pressed it yet. Once I press it what will happen is this fluid this liquid will be injected into this loop and it will just clean the loop and make it primed and ready for taking up the protein. So, what am going to do is, by this time I have completed flushing the entire tube system.

So, what am going to do is now set it up in the load mode. So, once I do this the buffer has now began traveling throughout this tubes and it is now actually traveling through the column; which is why you will see that earlier there was no pressure, but now of pressure value is being shown. So, there is a difference in pressure across the two ends of the column and it is 0.38 mega Pascal at the moment.

So, this is within our safety limit of 0.5 mega Pascal which is why the machine is continuing to run. And it is indicating that everything is with the system as of now. Right now what am going to do is am going to flush the protein loop as well. So, I have gradually steadily injecting my buffer into the loop and the loop is there by getting cleaned by this new buffer so I have done that.

And right now as you can see the machine is on, but nothing much has happened that is because we have not actually introduced anything into the machine yet. Now finally, what I am going to do is; I am going to load my protein mixture into this loop so, yes.

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So, now, I am going to load my load my proteins mixture into this loop. So, he has the filtered protein solution mixture. And be careful I do not want to poke him or myself with the needle. So, so yes I need to be gentle I do not want thoughts of bubbles to be introduced. Sometimes when you are working with very concentrated solutions of proteins some amount of protein is inevitable.

But still try to minimize bubble formation as much as possible that is good for your experiment and good for the protein that you have. So, now, I have my protein in the syringe I will just get rid of minor bubbles so that is fine I think it is ok. Yes, you should be very paranoid when you are talking about bubbles particularly when using size exclusion chromatography columns because if you introduce bubbles into the matrix, you will end up damaging your column.

So, I have 2 ml slightly more than 2 ml of protein. So, now, what am going to do is I am going to introduce it into the loop. So, as I keep pushing it am basically now introducing protein mixture into this loop and the earlier buffer which I had cleaned the loop is now being displaced by this protein mixture. So, now, that I have done this, so now I am actually ready to start the actual separation process, my protein is loaded onto this loop and it is ready for separation.

All I have to do is change the valve orientation. So, subsequently the protein which is located here will be pumped via this loop path on to the column and then get separated

by the principle of size exclusion chromatography. So, right now what we have done is we have injected the protein into the protein loop. And next what we are going to do is we are going to change the valve system.

So, that the protein is now loaded onto the column. So, in order to do that what am going to do is, I am going to go to the settings and then set the position for the valve. So, initially it was set into such a position where the buffer from this container was flushing the entire tubing system and then going to waste after that I changed the mode into load mode. So, then the buffer that was kept here was being loaded onto the column and it was passing through the column which is why we are seeing a pressure difference in the display unit.

And now what am going to do is I am going to set it into a different mode which is called as inject mode. And what this does is it will now read out the buffer additionally through this loop and as a result it will inject the protein from here it will carry the protein and take it to the top of the column. And then that will actually start the process of separation by size exclusion chromatography.

So, I am setting it up to the inject mode and up to 0 here. And now what you are going to see is that the protein is being now carried into the column. So, the whole protein mixture is now being pumped to the top of the column. And after that it will be separated on the basis of the principles of size exclusion chromatography. Meanwhile buffer is also continuously being pumped through the column at a constant flow rate of 1 ml per minute as I have set up already.

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And yes, as you can see here now as soon as I started injecting it there is some initial changes in data which happens. But gradually you will see that it will flat line and it will continue going at a steady place. Until and unless your protein starts diluting because once protein starts eluting you will have an absorbance at 280 which will increase these values. So, what value you are seeing now if I zoom in so it is around 0 right.

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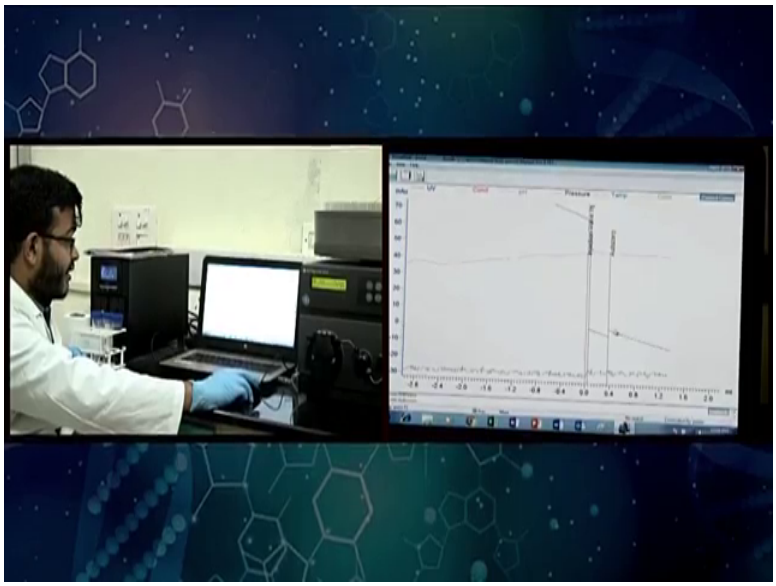

So since it is around 0 this is the absorbance that is coming from this buffer the machine has already taken that into account and it has removed that value. So, whatever absorbent that you will see will be coming solely from the proteins that are being eluted out and not

from the buffer. So, we have already started sending our protein through the column and it is been traveling through there.

So, now, what am going to do is, am going to start collecting the eluted liquid that is coming up through the column. So, what I am going to do is I am going to collect the liquid that is coming out in units of 1.5 ml. So, I have tubes here and am going to collect 1.5 ml of liquid in each tube and then it is going to rotate and go to the next tube. So, first I am going to adjust this so that. So, the liquid that is coming out will come out to this tube.

And it will be collected into these collection tubes in a drop wise manner I am just orienting it for the first time. Once I have already entered the first two directly below this nozzle and it is getting collected the other tubes the machine itself will take care of. So, am going to start collecting fractions of 1.5 ml each. And it might not be visible. But what is happening is now the liquid that was being carried from this buffer container through these tubing passing where the column.

And then passing through the detectors is now after passing through the conductivity detector and the absorbance UV a 280 detector. It is now passing through this tube tubing values being collected in these tubes. So, I have set it in such a manner so that once 1.5 ml of liquid has been collected this drum is going to rotate. And the next 1.5 ml will be collected in the next tube again it is going to rotate and then it is again going to collect.

So, all this rotation is fully automated I do not need to worry about it. All I need to ensure is the first time when I set it up I need to make sure that that tubing the outlet is perfectly aligned with the collection tube beneath. In a few short moments you will see once 1.5 ml of liquid has been collected it is going to adjust itself rotate and collect the next fraction. So, basically this is a fraction collector and it is collecting fractions.

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If you will notice the computer right now what you will see is this is the absorbance in the x axis and in the y axis you have the volume of liquid that has passed from the buffer container through the column. So, around 40 ml of buffer has passed and you can see it is more or less a straight line. And now you will see that it is starting to spike, I will zoom into it subsequently and show you later on, but it is starting to spike.

And since we are monitoring the absorbance at 280 this means that some component is now being eluted out from the column which can absorb at 280 nanometers. Since we are working with proteins and proteins most of them a large percentage of proteins have tyrosine, tryptophan, and phenyl alanine residues. These residues mostly absorb at 280 nanometer and as a result we are actually now measuring that some protein is now being eluted out and it is being collected in that tube.

I will zoom in and you can see that before the two eighty started coming out it was flat. And now as it is coming on there is a slight increase a very small amount of protein came out so the rise is not as much as you would have liked to see. But as you will as we will keep on going on the increase for the different proteins will become more pronounced. Also what I wanted to show you see here there is a number this is number 1, this is number 2, and numbers will keep on popping up.

So, these numbers actually indicate which tube contains what portion of sample. So, tube 1 actually contains liquid in, which very less amount of protein is present. But tube 2

once this will rotate and you will see that fractions are being collected in tube 2 you can actually identify that this region which seems to absorb a 280 is collected in tube two later on after the experiment you are going to separate out tube 2 to take apart a small amount of sample and run it on an SDS page to check for the presence of your protein.

And in case it is presence present you can also establish the purity of the protein that has been eluted. If you have noticed this turn right now and the next tube has been aligned with the outlet nozzle. So, now, this means that the first tube has collected 1.5 ml of the eluted liquid and the next tube is collecting the next 1.5 ml and so on and so forth. This machine can hold at a time close to 100 such collection tubes so you can actually collect a large volume of liquid using this technique.

I will just zoom it out so that you can see. Now that have zoomed out the data you can see quite clearly that this is flat lining, but here there is a small rise which means some amount of protein has eluted out. Notice that it has eluted out at around 40 to 45 ml. So, this is an information that I already know for the column that we are using for today's experiment it has a column volume of 120 ml. And it has a wide volume of 40 ml and you will be taught in theoretical classes what void volume means.

Basically void volume of a size exclusion chromatography column means this is the volume at which any component which could not enter any of the course of the matrix, but could travel only in the gaps between the different beads comes out. So, at 40 ml for this column I know that soluble aggregates which have a size of close to greater than 600 kilo Daltons are coming out.

So, this is not actually my desired protein because my protein has a smaller size. These are some soluble aggregate contaminants which had been present in the mixture. And I have actually successfully separated them out and collected them in tube number 2 and 3. As this experiment progresses you will keep on seeing that different peaks will come out or will show up corresponding to different protein components as and when they are being eluted.

So, our protein purification has been progressing since it is a very big column and it is column volume is 120 ml and I have set up the machine in such a way that the flow rate of buffer is 1 ml per minute. So, for buffer to completely enter the column at that end and be completely eluted out at this rate, it will take 2 hours. So, initially I had shown you at

40 ml a small peak being eluted which I had mentioned was the soluble aggregates that were coming out in the void volume.

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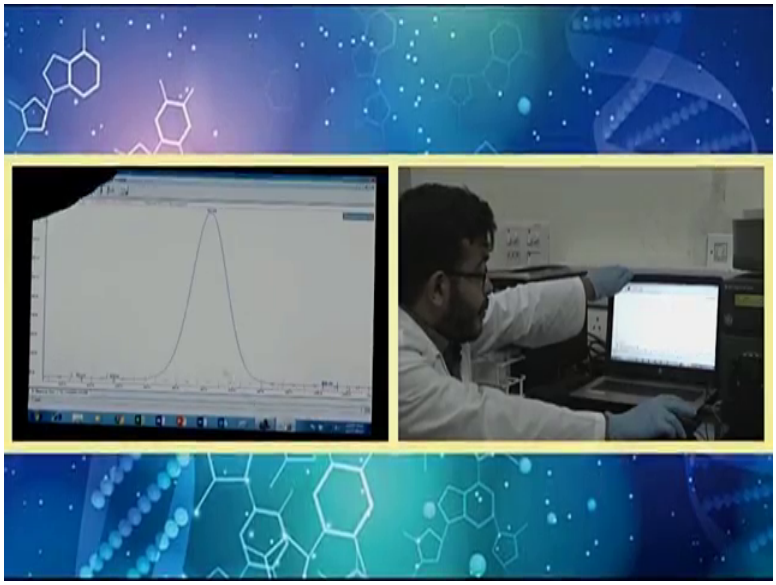

However, now I will show you the complete result what you can see here is in the x axis I have the eluted volume of buffer in ml and on the y axis I have absorbance at 280 as measured. So, you can notice once I zoom in I am zooming in this portion you see that at around 92 ml of elution volume you can see that there is a maximum of a very large peak which absorbs at 280. So, compared to that if I know zoom out here and go back to 40 ml or 42 ml where I had shown you a peak earlier.

You will notice that that peak seems to be just like the flat line. This means that because of the huge amount of this protein the scale in the axis renders this peak to be negligible. And you cannot really distinguish it from the basal absorbance of the buffer. But this peak that you see here it has a very symmetrical shape. So, what this means is in tube number 52 up till tube 50 or 51 as I can show you here only buffer was being eluted out. But at 52 a protein started eluting out because it was absorbing at a 280 we expect it to be a protein.

That is where we expect our protein to elute out and it keeps rising it keeps rising till it reaches a maxima and then it keeps going down. And from this sort of curve shape you can say that you have a very pure and homogeneous population of protein. What am next going to do is; see notice that this peak protein the proteins that are comprising this peak

have been collected in tubes number; 53, 54, 55, 56, and 57. So, I will I do not know if is possible to zoom in I will just zoom in further.

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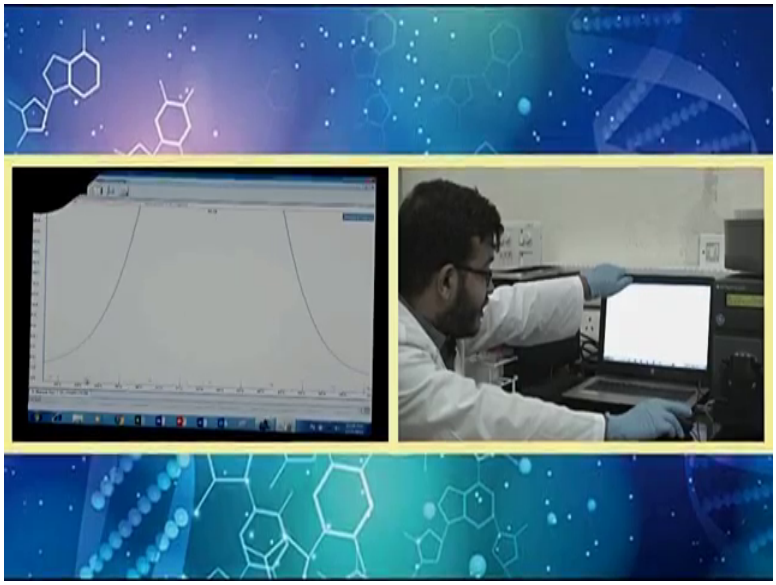

So, 53, 54, 55, 56, 57 contain peak contain the peak region of this protein. And what am going to do is each of these tubes holds 1.5 ml of protein. So, what am going to do is now collect these separate tubes and store them with me. And later on am going to take a small amount of sample from each tube and loaded on a protein gel on SDS page gel. And then am going to check for the presence of protein.

If I get a single band corresponding to the expected molecular weight of my protein and I do not get any other bands in the protein gel; then I can reasonably say that this fractions these tubes contain my desired protein of interest. And I have successfully removed other contaminants or soluble aggregates from that protein. Because even if there are other proteins of different sizes they are present in other tubes and they have been separated from the protein solution present in this region. This is how we purify proteins using size exclusion chromatography.